PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Patent application of Mortimer Civan et al.	
		Group Art Unit: 1614
Serial No.:	10/009,581	
Filed:	April 30, 2002	Examiner: Donna Jagoe
For:	Methods for Controlling IntraOcular Pressure	Conf. No. 1751

DECLARATION OF DR. MORTIMER M. CIVAN under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Mortimer M. Civan, declare that:

1. I am an inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 10/009,581, claiming for the first time that it is possible to regulate intraocular pressure in an eye by administering to the eye a pharamaceutical composition comprising a pressure-modulating amount of a sodium-hydrogen exchanger (NHE) inhibitor.

2. To facilitate submission of this Declaration, I have not attached a copy of my *curriculum vitae*. However, should my c.v. become required, I will produce it upon request. Briefly, however, my career has been devoted to studies of the basis for the formation of aqueous humor in the eye in order to better understand and treat clinical glaucoma. I am currently Professor of Physiology and Medicine, Department of Physiology at the University of Pennsylvania School of Medicine, and a Member of the Cell and Molecular Biology Graduate Group. I hold an M.D. degree from Columbia University, NY and an A.B. summa cum laude from Columbia College, NY with a major in chemistry . I have been honored with several awards, including being elected a Fellow of American Association for the Advancement of Science, selected as an Established Investigator for the American Heart Association, a Member of the American Society for Clinical Investigation, selected as Overseas Fellow at Churchill College, University of Cambridge, a Faculty Scholar of the Josiah Macy, Jr. Foundation,

recipient of the Dean's Award for Excellence in Teaching Basic Science, University of Pennsylvania School of Medicine, and appointed Harold Chaffer Memorial Lecturer on the Faculty of Medicine, University of Otago, Dunedin, New Zealand. In addition, I was editor of *The Eyes' Aqueous Humor: From Secretion to Glaucoma*, Academic Press, San Diego, CA, 1998, and have been invited by Elsevier Press to edit a new edition of that work.

3. I have read all of the communications from the U.S. Patent and Trademark Office (PTO) relating to the above-identified application, and I have participated in the Response process for each Office Action. I was also an active participant at the in-person Examiner's Interview for this application on October 27, 2005 and spoke with Examiner Jagoe at length on the subject of my invention and the comments made in the Office Action dated August 11, 2005, I am well qualified to discuss NHE inhibitors and beta blockers in regard to my claimed invention.

4. Claims 94-106 are pending in our application. Claim 94 is an independent claim, and all other pending claims are directly or indirectly dependent upon claim 94. Claim 94 reads as follows:

> 94. A method of regulating intraocular pressure by inhibiting sodium/hydrogen antiport activity in the eye, comprising administering to ciliary epithelial cells in an eye of a human or an animal having a trabecular network a pharmaceutical composition, wherein the pharmaceutical composition comprises a pressure-modulating amount of at least one sodium-hydrogen exchanger (NHE) inhibitor.

5. The class of molecules designated "NHE inhibitors" are known in the art. They have been, and continue to be, used in cardiovascular therapies. At the time of the invention, NHE inhibitors did not include timolol or any other beta blocker. For instance, Karmazyn discloses a variety of NHE inhibitors (*Ann. N.Y. Acad. Sci.* 874:326-334 (1999)). See pp. 326-327, bridging sentence. Karmazyn does not disclose or suggest timolol or any other beta blockers are NHE inhibitors. Subsequent to the invention, NHE inhibitors still do not include beta blockers. For instance, Masereel *et al.* review numerous NHE inhibitors but do not teach or suggest beta blockers are NHE inhibitors (*Eur. J. Med. Chem.* 38:547-554 (2003)). Thus, the term "NHE inhibitors" does not refer to or suggest beta blockers.

6. In our instant application, timolol is identified properly as a beta blocker. See for instance, p. 3, lines 25 and p. 4, lines 19. Therefore, claim 94 does not embrace timolol or any

other beta blockers. There is no definitive scientific proof that timolol acts on the NHE antiport of the eye.

7. Our claimed invention is one in which administration of a pressure-modulating amount of an NHE inhibitor to an eye, having a trabecular network, regulates intraocular pressure. No one in the art demonstrated or knew, before my present invention, that the antiport inhibitors *per se* lower intraocular pressure, and further control the uptake and release of salts within the aqueous humor of the eye. As a result the methods drawn to the use of the NHE inhibitors in our invention are, in fact, both novel and nonobvious.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,

Date: November 9, 2005

Mortimer M. Civan

The Role of the Myocardial Sodium-Hydrogen Exchanger in Mediating Ischemic and Reperfusion Injury

From Amiloride to Cariporide[a]

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ABSTRACT: There is convincing evidence that the Na-H exchanger (NHE) plays a pivotal role in mediating tissue injury during ischemia and reperfusion. Extensive studies with NHE inhibitors have consistently shown protective effects against ischemic and reperfusion injury in a large variety of experimental models and animal species, particularly in terms of attenuating contractile dysfunction. These protective effects of NHE inhibition appear to be superior to other strategies, including ischemic preconditioning. Such studies have contributed greatly to the overwhelming evidence that NHE activation mediates ischemic and reperfusion injury. The NHE inhibitor HOE 642 (cariporide) is currently undergoing clinical evaluation in high-risk cardiac patients. Moreover, there is now emerging evidence that NHE may be involved in mediating cardiotoxicity directly produced by various ischemic metabolites such as lipid amphiphiles or reactive oxygen species. NHE inhibition also attenuates apoptosis in the ischemic myocardium, a process that may be of importance in the subsequent development of postinfarction heart failure. In conclusion, NHE represents an important adaptive process in response to intracellular acidosis that results in a paradoxical contribution to cardiac tissue injury.

NHE AND THE ISCHEMIC MYOCARDIUM: A BRIEF HISTORICAL PERSPECTIVE

NHE represents one of the major pH regulatory systems in the cardiac cell. The concept that this system may be involved in cardiac pathology was first proposed by Lazdunski and coworkers in 1984[1] based on the observation that sodium influx concomitant with proton efflux may produce undesirable effects through disordered calcium homeostasis. The first experimental evidence came from the author's laboratory in 1988,[2] where it was reported that amiloride, a relatively non-specific NHE inhibitor, protected the ischemic and reperfused myocardium. Since that initial observation dozens of studies have reported on the protective effects of NHE inhib-

[a]Studies from author's laboratory are supported by the Medical Research Council of Canada. The author is a Career Investigator of the Heart and Stroke Foundation of Ontario.

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itors including more selective amiloride analogues and the more recently developed compounds HOE 694 and HOE 642 (cariporide). It has been particularly impressive that virtually all reports from a variety of laboratories have demonstrated protective effects. These impressive findings formed the basis for the rapid approval and establishment of a multicentered international clinical trial, the GUARDIAN study, to assess the effects of cariporide in high-risk patients with acute coronary syndromes. The four major group of patients recruited into this study include (1) patients with unstable angina, (2) patients with non–q wave myocardial infarction, (3) unstable patients requiring percutaneous transluminal coronary angioplasty, and (4) unstable or high-risk patients requiring coronary artery bypass surgery. The two primary endpoints are myocardial infarction and mortality. The results of the trial are expected in 1999. Taken together, these developments represent rapid progress in the development of novel strategies in cardiovascular therapeutics that will hopefully result in a reduction in morbidity and mortality in patients with heart disease.

INTRODUCTION: pH_i REGULATION AND NHE

Changes in intracellular pH (pH_i) can produce marked effects on cardiac contractility, particularly acidosis-induced negative inotropic effects. Although the mechanisms involved in pH-regulated contractility are very complex, they reflect the direct interfering effects of protons on various cellular processes involved with excitation-contraction coupling.[3] It is therefore critical that the cell possesses mechanisms by which pH_i is regulated especially after intracellular acidosis as a consequence of myocardial ischemia. Two major alkalinizing exchangers exist in the cardiac cell, the NHE and a Na-HCO_3^- symport. The NHE represents one of the key mechanisms for restoring pH_i following ischemia-induced acidosis by extruding protons concomitantly with sodium influx. The simultaneous entry of sodium during NHE activation indicates that this process is also an important route for increasing intracellular sodium concentrations during various conditions and represents the major mechanism postulated to mediated NHE-dependent cardiac injury through modulation of intracellular calcium levels, as discussed further below. To date, at least five (a mitochondrial NHE-6 isoform has been identified although its function is unknown) distinct isoforms of the exchanger have thus far been identified that possess structural differences as well as varying sensitivities to inhibition by pharmacological agents. It appears that the ubiquitous subtype 1 (NHE-1), a glycoprotein with a molecular weight of approximately 110 kD, is the predominant isoform in the mammalian myocardium. NHE-1 comprises two major functional domains: a hydrophobic region that spans the membrane 12 times and that is critical for exchange activity, and a hydrophilic moiety that is likely of importance for hormonal modulation of the exchanger. Further details concerning the molecular structure and regulation of the NHE can be found in a number of recent review articles and monographs.[4–6] The activity of NHE-1 can be modulated by a number of growth factors, hormones, and neurotransmitters (via various kinases, including tyrosine kinases, Ca-calmodulin kinase, MAP kinases, and PKC coupled to G proteins), as well as by hypertonic shrinking and mechanical stimuli. Most of these signals stimulate NHE by shifting the pH_i-activity curve towards the alkaline range, thus stimulating the enzymatic activity of the transporter at constant pH_i and moving

TABLE 1. Beneficial effects of Na-H exchange inhibitors on the ischemic and reperfused heart

Enhanced/accelerated systolic recovery after reperfusion
Diminished ischemic and reperfusion-induced contracture
Reduced arrhythmias including supression of postinfarction ventricular fibrillation
Prevention of postinfarction mortality
Reduced calcium and sodium overload
Preservation of energy metabolites
Reduced necrosis
Reduced apoptosis
Reduced toxicity of various ischemic metabolites and paracrine/autocrine factors

it closer to its maximal rate.[7] Conversely, inhibition of the exchanger can be accomplished by a variety of drugs, the prototypical being amiloride and its *N*-5 disubstituted derivatives.[8] Recently, the benzoyl guanidinium compounds 3-methylsulfonyl-4-piperidinobenzoyl-guanidine methanesulfonate (HOE 694) and 4-isopropyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE 642) have been shown to be effective NHE inhibitors. HOE 642 (cariporide) is of particular interest as it appears to be a selective inhibitor of the NHE-1 isoform, the primary if not sole subtype found in heart, rendering it particularly attractive for therapeutic intervention in cardiac disorders while minimizing the potential for side effects.[9]

EFFECTS OF NHE INHIBITORS ON THE ISCHEMIC AND REPERFUSED MYOCARDIUM

Evidence in the literature strongly supports the concept that NHE inhibition bestows excellent myocardial protection against ischemic and reperfusion injury. As discussed in a number of recent reviews[6,10–12] and as summarized in TABLE 1, these salutary effects have been demonstrated on numerous parameters of cardiac function, including enhanced contractility, reduced contracture, and a decrease in the incidence of arrhythmias. In addition, improvements in biochemical and ultrastructural indices have been extensively demonstrated with NHE inhibition. Such protection has been demonstrated with the amiloride series of agents and with both HOE 694 and HOE 642. This protection is associated with diminished tissue sodium and calcium content, in support of a close association between NHE and Na-Ca exchange activity.[13,14] Indeed, reduction in calcium overload appears to represent a major mechanism of action of NHE inhibition (see below).

INTERACTION BETWEEN NHE INHIBITORS AND OTHER CARDIOPROTECTIVE STRATEGIES

Studies have been done to ascertain potential interactions between NHE inhibitors and other pharmacological agents or approaches used to protect the myocardium. Such interactions are of potential importance as they may be relevant when utilizing multifaceted approaches towards myocardial protection. For example, it

has been shown that amiloride enhanced the protection afforded by reduction of extracellular sodium and calcium concentrations in isolated working rat hearts subjected to 30 min of cardioplegic arrest.[15] Moreover, in isolated working rat hearts subjected to ischemia, administration of amiloride in combination with the hydroxyl radical scavenger desferrioxamine produced superior cardioprotective effects compared to each drug alone.[16] These results are therefore suggestive of a specific and distinct target for the beneficial effects of NHE inhibition rendering this approach attractive for potential superior cardioprotective strategies using drug combination protocols. Moreover, we have recently demonstrated additive protective effects of HOE 642 when the drug is administered in combination with either of the volatile anesthetics sevoflurane or isoflurane in isolated ischemic and reperfused rat hearts.[17] Although indicative of distinct mechanisms of action, these findings also suggest that the combination of HOE 642 and these volatile agents produces superior cardioprotection, which may be of importance under clinical conditions where effective cardioprotection is desired during surgical procedures.

IS NHE INHIBITION A SUPERIOR CARDIOPROTECTIVE STRATEGY? COMPARISON WITH ISCHEMIC PRECONDITIONING

Detailed comparisons between NHE inhibition and other modes of cardioprotection have not been extensively studied yet these types of studies are important in order to design the most effective therapeutic approaches. Work in our laboratory has compared ischemic preconditioning with NHE inhibition with cariporide in hearts subjected to increasing periods of zero-flow ischemia followed by 30-min reperfusion. Ischemic preconditioning was carried out with two 5-min cycles of ischemia separated by a 10-min reperfusion before initiating prolonged ischemia. Alternatively, experiments were also done in the presence of 5 μM cariporide. The essence of our findings is that improved recovery of function is similar with the shorter ischemia durations although cariporide-treated hearts recovered significantly faster. The most marked observation was that cardioprotective effects of cariporide were still evident when ischemia was extended to 90 min, whereas no salutary effects were seen with ischemic preconditioning. Accordingly, it appears that NHE inhibition is advantageous and superior to ischemic preconditioning, particularly under conditions of prolonged ischemia when the effects of the latter are no longer evident.

MECHANISMS OF NHE INVOLVEMENT IN ACUTE ISCHEMIC AND REPERFUSION INJURY

A number of concepts are emerging regarding the mechanisms underlying NHE involvement in the ischemic myocardium and it appears that NHE may be involved in multiple mechanisms. FIGURE 1 outlines these concepts and shows that these mechanisms are interrelated, suggesting that although calcium is the final mechanism, the activation of NHE and the role of the antiporter in mediating injury likely involve multifaceted aspects.

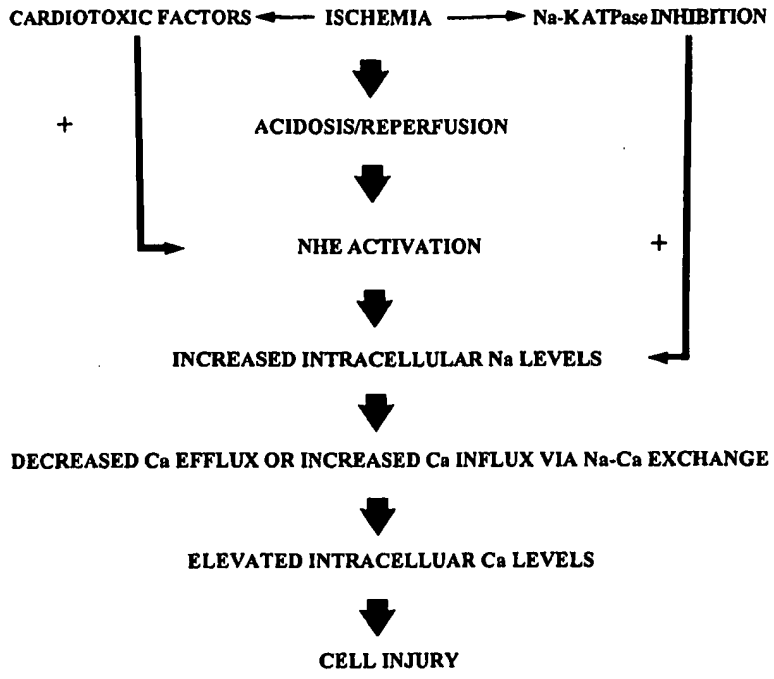

FIGURE 1. Pathways of NHE activation and its involvement in acute responses to myocardial ischemia and reperfusion. See text for details.

Modulation of Intracellular Calcium Levels

The role of calcium is based on the proposal that reintroduction of flow to the previously ischemic, and thereby acidotic, cardiac cell establishes a rapid transsarcolemmal proton gradient resulting in activation of NHE. This has been reviewed in several publications.[1,6,10–12,14] While this would contribute to restoration of pH_i, the concomitant sodium influx could result in increased intracellular calcium concentration via the Na-Ca exchanger due to reduced calcium efflux resulting from the reduction of the sodium gradient driving the Na-Ca exchanger. Moreover, it should be emphasized that in the ischemic cardiac cell Na-K ATPase is inhibited resulting in an elevation in intracellular sodium concentration due to reduced ability to extrude sodium. Activation of NHE, particularly upon reperfusion, which in itself is greater due to the prior ischemia-induced accumulation of protons, provokes a greater elevation in intracellular sodium and calcium concentrations particularly under conditions of defective ion regulatory mechanisms. The net result of large elevations in intracellular calcium levels coupled with intracellular alkalosis due to NHE-mediated proton extrusion is tissue damage manifested by intracellular calcium overload, contracture, and depressed systolic function. Moreover, there is also evidence, par-

ticularly that based on NMR studies, to suggest that NHE activation during ischemia per se, that is prior to reperfusion, also contributes to both calcium and sodium overloading, providing yet another contributing factor to cell injury.[18]

Attenuation of Cardiotoxic Effects of Ischemic Metabolites

The above hypothesis suggests that the primary mechanism for NHE activation is intracellular acidosis. However, further NHE activation occurs because of direct stimulation by metabolites produced by the ischemic myocardium. For example, levels of endothelin-1 (ET-1), a potent NHE activator, are elevated in myocardial ischemia and may produce deleterious effects on the reperfused myocardium, inducing both diastolic and systolic abnormalities.[19] We have shown that the toxicity produced by ET-1 can be attenuated by NHE inhibition, suggesting an important role of the antiporter in mediating the detrimental effect of the peptide on the ischemic and reperfused myocardium.[20] NHE activation may also represent an important mechanism for arrhythmogenesis in the reperfused myocardium particularly under conditions of elevated catecholamine levels. For example, although α_1 adrenergic agonists enhance ventricular arrhythmias in the reperfused myocardium this effect can be markedly decreased by NHE inhibition.[21]

We have recently shown that lysophosphatidylcholine, one of the predominant tissue metabolites that accumulates rapidly in the ischemic myocardium, is a potent NHE activator in the cardiac cell and that the cardiotoxic effects of this amphiphile, at least at low concentrations, can be markedly attenuated by NHE inhibitors.[22] In addition, it appears that at least some of the direct toxic effects of hydrogen peroxide can be attenuated by NHE inhibition.[23] Moreover, we have recently demonstrated that the ability of very low, sub-toxic concentrations of hydrogen peroxide to compromise postischemic ventricular recovery can be attenuated by NHE inhibition.[24] Taken together, these observations open up the possibility that a variety of intracellular factors produced during ischemia may contribute to tissue dysfunction through NHE-dependent processes.

Apoptosis

There is now increasing evidence that apoptosis or "programmed cell death" is an important response of the myocardium to ischemia. Apoptosis is rapid, precedes cell necrosis, and appears to contribute the overall sequelae of cardiac injury.[25–27] We recently demonstrated that HOE 642 significantly attenuated the development of early apoptosis in hearts subjected to 30-min global ischemia with or without reperfusion.[28] Moreover, dietary cariporide inhibits reperfusion-associated apoptosis in the acutely infarcted myocardium (unpublished data). This is clearly an important area for further study, particularly, as discussed below, since apoptosis is emerging as an important contributor to the postinfarction remodeling process leading to heart failure.

EVIDENCE FOR A ROLE OF NHE IN POSTINFARCTION RESPONSES

As evidenced from the preceding discussion, most studies on NHE involvement in heart disease have centered on acute responses. One study has shown that adding

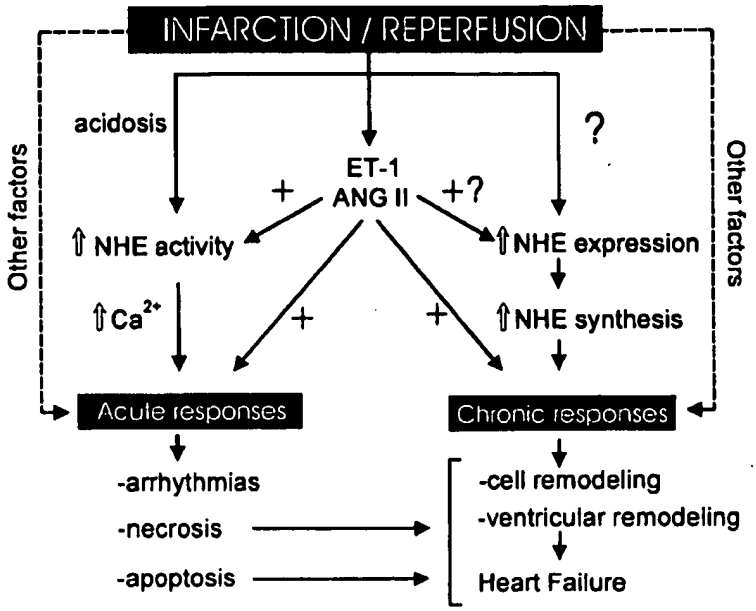

FIGURE 2. Role of NHE in the acute responses to myocardial ischemia and reperfusion and potential consequences in terms of chronic postinfarction responses. See text for details.

amiloride to the drinking water of rats with infarcted myocardium resulted in a significant attenuation of ventricular remodeling.[29] Preliminary data from our laboratory demonstrate that dietary cariporide completely prevents ventricular fibrillation and mortality in these animals and reduces other forms of arrhythmias. Moreover, the degree of apoptosis was decreased as was the ability of infarction to upregulate NHE-1 expression. The potential benefits of the antiarrhythmic effects of cariporide are obvious in terms of early management following myocardial infarction. Apoptosis has emerged as a potentially critical factor in the response to infarction[30] and evolution to heart failure.[31–33] Thus, the fact that cariporide can inhibit this phenomenon[28] further reinforces its potential usefulness in postinfarction responses. Although the relevance is unclear at present, increased NHE-1 expression postinfarction is intriguing and suggests that NHE influences not only acute, but also chronic postinfarction responses, the latter occurring through an as-yet undefined mechanism of upregulated NHE-1 expression. Paracrine/autocrine regulation by ET-1 or angiotensin II may contribute to this process. These concepts are summarized in FIGURE 2.

SUMMARY

There is now strong evidence that NHE activation in the ischemic and reperfused heart plays a major role in restoring pH_i, which at the same time contributes to tissue

damage most likely via a number of complex mechanisms. This concept is supported by the fact that virtually all studies thus far reported have demonstrated cardioprotective effects of NHE inhibitors. In addition to its role in acute ischemia and reperfusion, it is likely that the antiporter will also be found to be of importance in other scenarios of ischemic injury, such as that involving long-term cardiac preservation. The beneficial effects of NHE inhibitors coupled with the likely low toxicity of these agents, particularly with respect to the novel isoform-specific inhibitors, provide promise for the development of new strategies for the protection of the ischemic myocardium as well for the heart subjected to reperfusion procedures.

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Invited review

An overview of inhibitors of Na^+/H^+ exchanger

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Abstract

The Na^+/H^+ exchanger (NHE) is involved in intracellular pH homeostasis of many mammalian cell types. To date seven NHE isoforms (NHE1–NHE7) have been identified. NHE1 is the most predominant isoform expressed in heart where it contributes to cardiomyocyte pH homeostasis. Although the NHE activation is essential for the restoration of physiological pH, hyperactivation of NHE1 during ischemia–reperfusion episodes disrupts the intracellular ion balance, leading to cardiac dysfunction and damage. Beside its ability to inhibit a conductive Na^+ channel and the Na^+/Ca^{++} exchanger, amiloride was the first drug described as NHE inhibitor. Double substitution of the nitrogen of the 5-amino group of amiloride gave DMA, EIPA, MIBA and HMA. Later, several acylguanidines were prepared to selectively inhibit NHE1. The replacement of the pyrazine ring of amiloride by a pyridine ring or by a phenyl increased the potency and the NHE selectivity. The simultaneous replacement of the pyrazine ring by a phenyl, of the 6-chloro by a sulfomethyl led to drugs such as HOE-694, cariporide, eniporide and BIIB-513 which also selectively inhibited NHE1. In the last decade several bicyclic guanidines were prepared: zoniporide, MS-31038, SM-20220, SM-20550, SMP-300, KB-R9032, BMS-284640, T-162559, TY-12533, S-3226 or SL-591227. Extensive pre-clinical studies indicated that NHE inhibitors afford substantial protection in different animal models of myocardial ischemia (MI) and reperfusion, but the results of clinical trials involving eniporide and cariporide were mixed.

Keywords: Sodium-proton exchanger; NHE; Amiloride; Inhibitor; Ischemia–reperfusion

1. Introduction

The Na^+/H^+ exchanger (NHE) is a protein that is expressed in many mammalian cell types. NHE is responsible for intracellular pH and cell volume regulation by extruding protons from, and taking up sodium ions into cells. To date seven isoforms (NHE1–NHE7) have been identified and cloned. NHE isoforms share ca. 20–60% amino acid identity and a molecular mass from 74 to 99 kDa (Table 1). NHE6 and NHE7 are localized to recycling endosomes and to the *trans*-Golgi network respectively, whereas the other isoforms (NHE1–5) are expressed in the cell membrane [1–3]. NHE isoforms are composed of 12 helical hydrophobic membrane-spanning segments, a N-terminal sequence and a highly hydrophilic C-terminal segment. The segments M3–M12 share a high sequence homology among the various isoforms where M6 and M7 are most highly preserved (95% identity), suggesting that these domains are involved in the transport of Na^+ and H^+ across the membrane [1].

NHE1 is activable by growth factors and expressed in several cell types, mainly in mammalian cardiomyocytes, platelets and on the basolateral membrane of renal tubules [4,5]. NHE2 has been localized in the gastrointestinal system mainly in stomach, colon and small intestine, with lower levels in skeletal muscle and in selected nephron segments [6–8]. Some studies reported basolateral and other apical localization of NHE2 [1]. NHE3 is mainly expressed at high levels in colon, and small intestine, with significant levels also in kidney and stomach [9,10]. It contributes to sodium absorption by the brush-border membrane in intestinal or renal epithelia. NHE4 is highly abundant in stomach and also present at intermediate levels in small intestine and colon [11]. A lower concentration is found on the

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Table 1
Isoforms of the Na^+/H^+ exchanger

Isoform	Species	Structure		Localisation
NHE1	Human	815	91 kDa	Cardiomyocytes, platelets Basolateral membrane of several tissues
NHE2	Rat	813	91 kDa	Stomach, colon, small intestine, adrenal gland
	Rabbit	809	90 kDa	Kidney and intestinal epithelia cell
NHE3	Rat	831	93 kDa	Colon, small intestine, stomach apical membrane of epithelia (proximal tubule, intestine)
NHE4	Rat		81 kDa	Stomach, small intestine, colon, collecting tubule
NHE5	Human	896	99 kDa	Brain (hippocampus), spleen, testis, skeletal muscle
NHE6	Human	669	74 kDa	Brain, skeletal muscle, heart
NHE7	Human	725	80 kDa	Brain (putamen, occipital lobe), skeletal muscle Secretory tissues (stomach, prostate, pancreas, thyroid)

basolateral membrane of collecting tubule. Little is known about the role of NHE5 which is expressed predominantly in nonepithelial tissue such as brain (hippocampus, cortex) [12–14]. NHE5 has been identified at a lower level in spleen, testis and skeletal muscle. In contrary to NHE1–5, NHE6 is the first intracellular NHE. It has been identified on recycling endosomes but not in the inner membrane of mitochondria as primarily assessed [2,15]. NHE6 has been detected with highest abundance in brain and skeletal muscle, followed by heart and other tissues. NHE6 may regulate intravesicular pH and contribute to lysosomal biogenesis. Finally, NHE7 has been localized predominantly to the *trans*-Golgi network [3]. Its expression is ubiquitous but predominant in certain regions of brain (occipital lobe, putamen), in skeletal muscle, in stomach and in glands (pancreas, salivary-, thyroid- and mammary-gland ...).

NHE is working according to the Na^+ and H^+ gradients by exchanging an extracellular Na^+ (Na_o^+) against a intracellular H^+ (H_i^+) with a tightly coupled 1:1 stoichiometry. NHE1-3 and NHE5 exhibited a hyperbolic dependence on Na_o^+ concentration ($[Na^+]_o$) while NHE4 showed a sigmoidal dependence on $[Na^+]_o$. The affinity of these different NHE isoforms for $[Na^+]_o$ is ranging between 5 and 50 mM (Table 2) [1]. Extracellular Li^+ (Li_o^+) and H^+ (H_o^+) competitively inhibit Na_o^+ influx by interacting at a single binding site of NHE1–3 and NHE5. In contrast to NHE2–3 and NHE5, extracellular K^+ (1–100 mM) inhibits NHE1 (Ki = 180 mM) at high and nonphysiological concentrations [14,16]. Only NHE7 and NHE4 are able to mediate the influx of K^+ or Na^+ in exchange for H^+ [3,17]. The decrease of intracellular pH enhanced the $[H_i^+]/[H_o^+]$ gradient and activates NHE isoforms (Table 2) [18]. In absence of Na_o^+, NHE operates in a reverse mode by expelling Na^+.

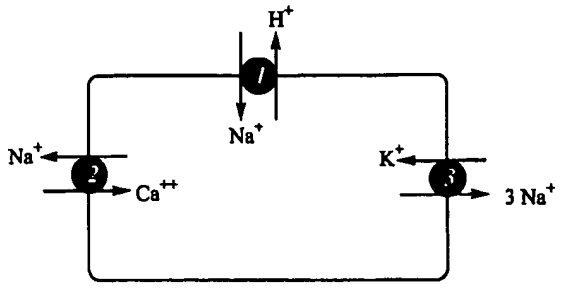
Table 2
Apparent affinity constants of rat NHE1-3 and human NHE5 isoforms for intra and extracellular monovalent cations

Cation	Apparent affinity constants ($K_{0.5}$) [a]			
	NHE1	NHE2	NHE3	NHE5
Na_o^+ (mM)	10.0	50.0	4.7	18.6
Li_o^+ (mM)	3.4	2.2	2.6	0.32
K_o^+ (mM)	19.5	None	None	Slight inhibition
H_o^+ (pK)	7.00	7.90	7.00	8.13
H_i^+ (pK)	6.75	6.90	6.45	6.43

[a] Values from Refs. [14,16].

NHE activity is regulated by several mechanisms [1]. Regulation of activity can be explained by direct phosphorylation of NHE by PKA and/or PKC [19]. NHE1 has been found to be constitutively phosphorylated in resting cells, and further phosphorylation is induced by phorbol esters, growth factors or phosphatase inhibitors [20]. The phosphorylation sites were detected on the distal part of the cytosolic C-tail. This NHE1 cytosolic tail contains also two calmodulin binding sites. Deletion of this segment constitutively stimulates NHE1 and mimics elevated intracellular $[Ca^{++}]$. The unoccupied domain, able to bind calmodulin with high affinity, exerts an autoinhibitory effect [21]. The binding of a calcineurin homolog protein (CHP) to a NHE1 sequence located on the C-tail inhibits the NHE1 activity. CHP appears to be constitutively phosphorylated [22]. For NHE2, two proline-rich domains that resemble SH3-binding proteins have been identified in the C-tail region [23]. NHE activity is also regulated by GTP-binding proteins. Activated forms of $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ activate NHE [24]. Recently, it has been evidenced that nitric oxide inhibited NHE3 activity via activation of soluble guanylate cyclase, resulting in an increase in intracellular cGMP levels and activation of protein kinase G [25].

Following intracellular acidosis, NHE activation is essential to restore physiological pH by H^+ extruding. Nevertheless, an excessive stimulation of NHE results in an increase of intracellular Na^+ concentration and a



1. Na^+/H^+ exchanger (NHE)
2. Na^+/Ca^{++} exchanger (NCX)
3. Na^+/K^+ ATP-pump

Fig. 1. Activation of NHE in ischemia–reperfusion: ischemia–reperfusion increase the pH_o/pH_i gradient which activate the NHE, enhance the activity of the Na^+/K^+ ATP pump, and of the Na^+/Ca^{++} exchanger leading to intracellular Ca^{++} accumulation.

subsequent activation of Na^+/K^+ ATPase, with a consecutive increase of energy consumption. The high intracellular Na^+ level contributes to activate the sarcolemmal Na^+/Ca^{++} antiporter which lead to raised intracellular Ca^{++} (Fig. 1).

At the cardiac level, this cellular Ca^{++} overload subsequent to NHE-1 activation is involved in ischemic and reperfusion injuries like myocardial infarction activation, stunning and tissue necrosis [26]. It has been demonstrated that the activity of NHE-1 is also increased in red blood cells, platelets, leukocytes, and skeletal muscle cells from patients with essential hypertension [27–31]. In response to chronic or acute hypertension, NHE-3 is redistributed from the apical brush border of proximal tubules to intermicrovillar and endosomal stores [32]. Finally, insulin induced a significantly increased NHE1 activity in normal patients as compared to obese individual were erythrocytes are resistant to insulin [33].

2. NHE inhibitors

With the aim to attenuate the harmful consequences of excessive NHE activation, several inhibitors were developed with the primary goal to provide cardioprotective drugs by inhibiting the NHE1 subtype. Beside its ability to inhibit a conductive Na^+ channel and the Na^+/Ca^{++} exchanger, amiloride, a K^+-sparing diuretic, was the first drug described as NHE inhibitor [34]. NHE1 and NHE2 are the most sensitive isoforms to amiloride inhibition whereas NHE3 and NHE4 are amiloride resistant isoforms [17] (Table 3). NHE5 is inhibited by amiloride at half concentration that was intermediate to those determined for NHE1 and NHE3 isoforms, but closer to the latter [14]. The latest isoform, NHE7 is insensitive to amiloride [3]. Cimetidine, harmaline and clonidine were also reported as weak and non-specific NHE inhibitors [35]. To increase the potency and the selectivity of inhibitors towards the NHE isoforms, and particularly NHE1, several molecules derived from amiloride have been synthesized and investigated. Double substitution of the nitrogen of the 5-amino group gave DMA, EIPA, MIBA and HMA, the most studied pyrazines related to amiloride (Fig. 2). EIPA, HMA and DMA are much more effective than amiloride on each studied isoform and lost the inhibitory potency on Na+ channel and Na^+/Ca^{++} exchanger (Table 3). They are weak selective inhibitors of NHE1. The selectivity of EIPA is ranging as follow: NHE1 > NHE2 > NHE5 > NHE3.

The pyrazine ring of amiloride was then replaced by a pyridine ring or by a phenyl. The phenyl counterpart of amiloride and the pyridine counterpart where the heterocyclic nitrogen was located in *meta* position of the acylguanidine were 54- and 36-times more active than amiloride on human platelet NHE1, respectively [36]. In the same experimental conditions, the pyridine derivative where the heterocyclic nitrogen was in *ortho* position of the acylguanidine was as active as amiloride [36]. Concomitantly to the replacement of the pyrazine ring of amiloride by a phenyl, the 6-chloro has been substituted by a sulfomethyl and the 2-amino has been deleted or replaced by a methyl group. Taken together, these modulations led to benzoylguanidines such as HOE-694 [37], cariporide [38], eniporide [39] and BIIB-513 [40] which completely lost the Na^+/Ca^{++} exchanger inhibitory potency as well as their ability to block Na^+-channels (Fig. 2). For each isoform investigated, HOE-694 is less active than EIPA but more selective towards NHE1 (Table 3). As compared to their inhibitory potency of NHE2, cariporide and eniporide are more NHE1-selective than EIPA. They are inactive on NHE3 and NHE5. As observed for the pyrazine derivatives, the substitution of the distal nitrogen of the acylguanidine moiety strongly decreased the NHE inhibitory potency. Later, several molecules based on a bicyclic template have been designed (Fig. 3). This bicyclic ring was a quinoleine (zoniporide [41], MS-31038 [42]), an indole (SM-20220 [43], SM-20550 [44], SMP-300 [45]), a benzoxazinone (KB-R9032 [46]), a dihydrobenzofurane (BMS-284640 [47]), a tetrahydronaphtalene (T-162559 [48]), or a tetrahydrocycloheptapyridine (TY-12533 [49]). Excepted for T-162559, all these compounds bear an unsubstituted acylguanidine group. Miscellaneous compounds structurally far from amiloride were also prepared (S-3226 [50], SL-591227 [51]) (Fig. 3). Enzymatic studies assessed that zoniporide, BMS-284640, (S)-T-162559 and SL-591227 were selective NHE1 inhibitors as compared to other isoforms (Table 3), whereas SM-20220, SM-20550 and TY-12533 inhibit at least NHE1. Finally, S-3226 was the first NHE3 selective inhibitor.

Table 3
Inhibitory potency of NHE inhibitors towards the different isoforms

Drug	Inhibitory potency (IC_{50} or K_i, in μM)[a]					
	NHE1	NHE2	NHE3	NHE4	NHE5	NHE7
Amiloride	*1–1.6**	*1.0***	*> 100**		*21*	
	5.3*		100–309*	813*		> 2000
EIPA	*0.01*–0.02***	*0.08*–0.5***	*2.4**		*0.42*	
	25.1*		3.3*	> 10*	1.53	
HMA	*0.013**		*2.4**		*0.37*	
DMA	*0.023**	*0.25**	*14**			
HOE-694	*0.085**		*640**		*9.1*	
Cariporide	0.03–3.4	4.3–62	1– > 100		> 30	
Eniporide	0.005–0.38	2–17	100–460		> 30	
Zoniporide	0.059	12	> 500*			
SM 20550	0.010*					
BMS-284640	0.009	1800	> 30		3.36	
T-162559 (S)	0.001	0.43	11			
T-162559 (R)	35	0.31	> 30			
TY-12533	0.017					
SL-591227	0.003	2.3				
S-3226	3.6	80**	0.02			
Harmaline	*140**	330	*1000**		*940*	
Cimetidine	*26**	330	*6200**		*230*	
	51*		> 1000*		> 1000*	
Clonidine	*210**	42	*620**		N.A.	

* = from rat, ** = from rabbit. NA = not active. Values are from references [3,7,14,16,17,41,45,47–51,59].
[a] K_i values are in italic.

The inhibitory potency of amiloride and some derivatives is reduced by high concentrations of Na^+, suggesting that their cationic form bind to the external Na^+ binding site. The potency of NHE inhibitors is not only related to the chemical structure but also to the ionization of the guanidine function. At physiological pH (7.4), the acylguanidine of amiloride ($pK_a = 8.78$) and the aminoguanidine of TY-162559 ($pK_a = 8.4$) are completely protonated, and can interact with NHE under their cationic form [48,49]. During ischemia or reperfusion phase, the pH falls down to 6.2. In these conditions, drugs like cariporide ($pK_a = 6.28$), TY-12533 ($pK_a = 6.93$) or zoniporide ($pK_a = 7.2$) are positively charged and then more efficient [41,49]. Indeed, cariporide and TY-12533 are more active at pH 6.2 than 6.7 (cariporide: $IC_{50} = 22$ nM/120 nM; TY-12533: $IC_{50} = 17$ nM/32 nM). This is also confirmed by the weak activity of the acylguanidine counterpart ($pK_a = 6.2$; $IC_{50} = 210$ nM) of the aminoguanidine TY-162559 ($pK_a = 8.4$; $IC_{50} = 9$ nM) [48].

3. Cardioprotective activity of NHE inhibitors

During myocardial ischemia, mitochondrial ATP synthesis ceases and glycolysis results in the depletion of ATP and in a decrease in intracellular pH which activates the NHE resulting in the extrusion of H^+ and the influx of Na^+. Due to NHE activation and to Na^+/K^+ ATP-pump failure, the overload of intracellular Na^+ activates the Na^+/Ca^{++} exchanger which increases the cytosolic free calcium. The accumulation of intracellular Ca^{++} contributes to cellular damage resulting in arrhythmias and myocardial stunning. With reperfusion, extracellular H^+ rapidly decreases increasing the intracellular to extracellular H^+ gradient. This large H^+ gradient activates NHE which enhances intracellular Na^+ and lead, through the Na^+/Ca^{++} exchanger, to accumulation of Ca^{++} during reperfusion. This contributes to arrhythmia and myocardial contracture during the reperfusion period. As NHE1 is the predominant isoform in mammalian myocardium, the NHE1 inhibitors were investigated in several models of ischemia–reperfusion. Administered 15 min prior to a myocardial ischemia (1 h) induced by occlusion of the left anterior descending coronary artery in dogs, eniporide (0.75 mg kg^{-1}, iv) significantly reduced the infarct size and the area at risk [52]. The reperfusion period was 3 h. In the same model, BIIB-513 (0.75 mg kg^{-1}), administered either prior to ischemia or prior to reperfusion, reduced the infarct size, phase 1b arrhytmias and ventricular fibrillation induced by ischemia and reperfusion respectively [53]. Zoniporide (CP-597396), a highly soluble selective NHE1 inhibitor, was also investigated in a rabbit myocardial ischemia (0.5 h)–reperfusion (2 h) model. Infused 30 min before ischemia until the end of reperfusion phase, zoniporide reduced the infarct size to a greater extent than

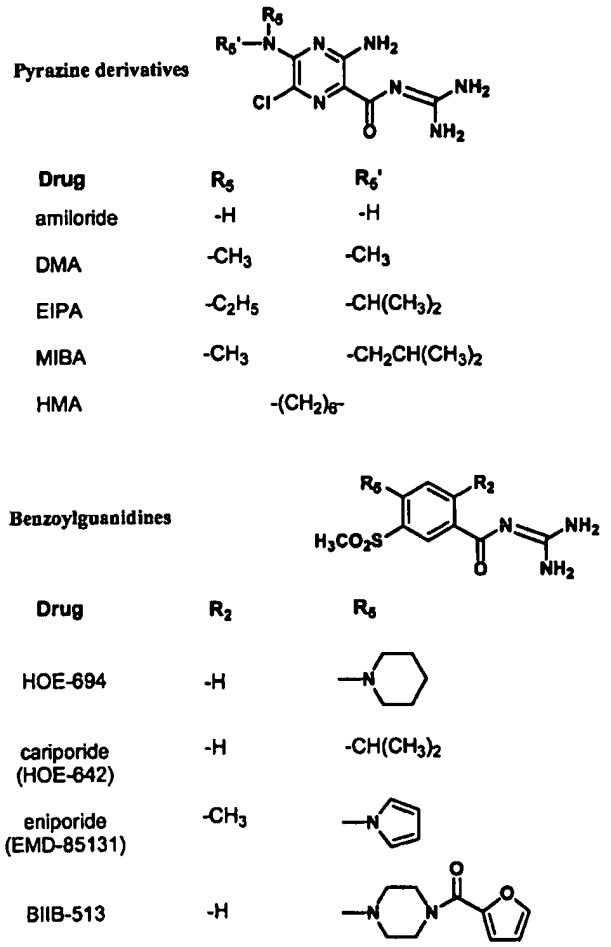


Fig. 2. Chemical structures of amiloride and of its pyrazine and phenyl derivatives.

eniporide or cariporide [54]. Furthermore, zoniporide did not cause any in vivo hemodynamic changes. In rabbit, SM-20550, an indole NHE-1 inhibitor which act on endothelial cells [55], 10 times more potent than EIPA, reduced the infarct size by ca. 30–70% in a dose-dependent manner (iv bolus 1.7–170 μg kg^{-1} followed by iv infusion 2.8–280 μg kg^{-1} h^{-1}) when administered prior to a myocardial ischemia–reperfusion (0.5–5 h) protocol [56]. Infused 10 min prior to the reperfusion period, the reduction was 20–40%. A similar experiment conducted in dogs showed that S-20550 (iv bolus 170 μg kg^{-1} followed by iv infusion 280 μg kg^{-1} h^{-1}) reduced the infarct size of 80 and 41% when administered 15 min prior to the occlusion of the left circumflex coronary artery (2 h) and to the reperfusion period (5 h), respectively [57]. Furthermore, SM-20550 suppressed ventricular fibrillation during both ischemia and reperfusion without affecting the size of the area at risk. SMP-300 inhibits NHE of rat myocytes with an IC_{50} of 6 nM and was therefore 16 times more potent than EIPA. SMP-300 (1 mg kg^{-1}), an orally active specific NHE inhibitor, reduced rat myocardial infarct size after 40 min of coronary artery occlusion followed by 24 h of reperfusion [58]. The cardioprotective effect of (S)-T-162559, a specific NHE1 inhibitor 5 and 31 times more active than eniporide and cariporide, respectively [59], was studied in a rabbit model of ischemia–reperfusion (0.5– 24 h) injury. Intravenously administered 5 min. prior to occlusion, (S)-T-162559 (0.03 and 0.1 mg kg^{-1}) reduced the myocardial area at risk by 36% [60]. The activity of TY-12533 was investigated in a rat model of myocardial ischemia–reperfusion (0.5–24 h), and compared to cariporide. Administered 5 and 10 min before the coronary snare occlusion and reperfusion respectively, TY-12533 and cariporide did not reduce the myocardial area at risk. The pre-occlusion treatment with TY-12533 and cariporide (0.1 mg kg^{-1} iv) reduced the infarct size of 50 and 70%, respectively. After a post-occlusion treatment, only TY-12533 (0.1 mg kg^{-1} iv) reduced the infarct size (44%). In dogs, TY-12533 (3 mg kg^{-1} 10 min^{-1}) injected 10 min before or after a myocardial ischemia–reperfusion (0.25–2 h) did not affect reductions in regional myocardial wall thickening and blood flow during ischemia, but it improved these parameters after reperfusion [61]. SL-591227 is the first potent and NHE1-selective non-guanidine inhibitor. In rat following left coronary artery occlusion (7 min) and reperfusion (10 min), SL-591227 (10–100 μg kg^{-1} min^{-1} iv) inhibited ventricular tachycardia (71–100%) and fibrillation (75–87%) induced by ischemia and reperfusion respectively. In rabbit, SL-591227 (0.6 mg kg^{-1} iv) reduced the myocardial area at risk (−58%) evoked by coronary occlusion and reperfusion (0.5–2 h) [51].

4. Cerebroprotective activity of NHE inhibitors

Three NHE isoforms (NHE 1, 4 and 5) have been found in brain tissues and are expressed in neurons and glial cells. As observed for myocardial infarction, brain ischemia–reperfusion activates NHE which increases intracellular Na^+, cellular swelling and free Ca^{++} accumulation leading to cellular damage. SM-20220 inhibited recovery from acid load in cultured neurons and glial cells with an IC_{50} of 5 and 20 nM, respectively [43]. The effect of SM-20220, a specific NHE inhibitor structurally close to SM-20550 (Fig. 3), has been studied in Mongolian gerbil global cerebral ischemia [62]. Transient ischemia (30 min) was induced by clipping both common carotid arteries, and SM-20220 was intravenously infused (0.3 or 1 mg kg^{-1}) immediately after reperfusion. For each dosage, SM-20220 improved the neurological outcome (McGraw's score) for 24 h, and significantly reduced the mortality rate at 1 mg kg^{-1}. SM-20220 and EIPA reduced free fatty acid from rat cerebral cortex during ischemia–reperfusion injury

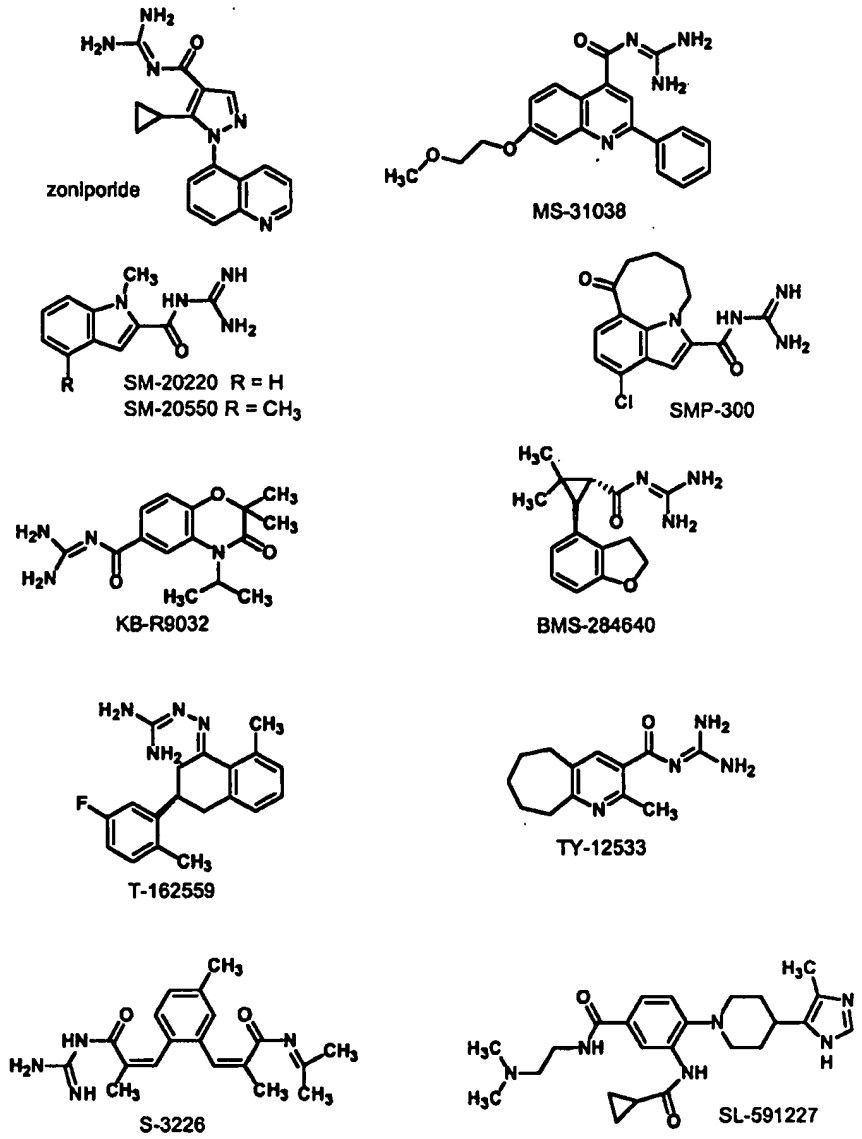


Fig. 3. Chemical structure of bicyclic NHE inhibitors.

[63,64]. These data indicate that these NHE inhibitors prevent the activation of phospholipases that occurs during reperfusion following a cerebral ischemia period. Finally, SM-20220, attenuated cerebral infract volume, water content and neutrophil accumulation at 72 h after permanent occlusion of the rat middle cerebral artery [65].

5. Renal protection of NHE3 inhibitors

Acute renal failure is characterized by sudden loss of the kidney function due to ischemia, trauma, and/or nephrotoxic drugs. As described for heart and brain, renal ischemia activates NHE, and particularly the NHE3 isoform which is expressed at high level in kidney. S-3226 is the first selective NHE3 inhibitor investigated in ischemia-induced acute renal failure in rats [66]. S-3226 (20 mg kg^{-1}, iv) infused before or after global renal ischemia (40 min) enhanced the creatinine clearance and reduced the increase of plasma creatinine as compared to the control. On day 7 following renal ischemia, kidneys revealed pronounced reduction of tubular necrosis, dilatation, protein casts and cellular infiltration when treated with S-3226.

6. Clinical investigations of NHE inhibitors

Extensive pre-clinical studies indicated that NHE inhibitors afford substantial protection in animal models of myocardial ischemia (MI) and reperfusion, with a

high level of conformity between different investigators, species and models. To date, results of clinical investigations with cariporide and eniporide have been reported in patients with evolving myocardial infarction and in those at risk of myocardial infarction [67–70]. The effects of cariporide have been evaluated in patients subject to anterior MI who were expected to receive perfusion therapy by primary coronary angioplasty within 6 h of the onset of symptoms [68]. Patients ($n = 100$) were randomized to receive placebo or cariporide (40 mg bolus iv) 10 min before reperfusion. Administration was completed within 4 h after the onset of symptoms. Cardiac enzymes and their isoforms (CK, CK-MB, LDH) were determined in blood samples taken before and after reperfusion (up to 72 h). Before treatment and at 3-week follow-up, contrast ventriculography was used to evaluate the left ventricular function. This study showed that the area under the curve of CK-MB release was reduced in the cariporide group as compared to the placebo group ($P = 0.047$). The ejection fraction was higher in the cariporide group than in the placebo one, such that the change from baseline to follow-up was greater in the latter group ($P = 0.045$). This study suggested that reperfusion injury could be a target for NHE inhibitors and these results warranted further clinical trials to confirm the therapeutic interest of NHE inhibitors. This led to the large-scale trial ESCAMI (Evaluation of the Safety and Cardioprotective Effects of Eniporide in Myocardial Infarction) [69]. This international, randomized, double-blind, placebo-controlled phase 2 trial enrolled 433 patients undergoing thrombolytic therapy or the percutaneous transluminal coronary angioplasty (PCTA) for acute ST-elevation MI to investigate the efficacy of eniporide on infarct size and clinical outcome. Eniporide was intravenously administered over 10 min. In patients receiving thrombolytic therapy the perfusion had to be completed at least 15 min after the start of treatment, while in patients subject to primary angioplasty, the infusion had to be completed at least 10 min prior to start PCTA. In stage 1 ($n = 430$), four doses of eniporide were considered: 50, 100, 150 and 200 mg. This stage had a triple goal: to evaluated the primary efficacy end point determined by cumulative release of α-hydroxybutyrate dehydrogenase (α-HBDH) and of cardiac markers (CK-MB, troponin T and I), to select a subset of doses to be carried forward in stage 2, and to determine the number of patients to be enrolled for stage 2. Within the first 6 weeks, death, cardiogenic shock, heart failure, arrhythmias, major bleeding were considered as secondary end points. In stage 1, the administration of 100 and 150 mg eniporide resulted in smaller enzymatic infarct sizes, especially in angioplasty group. In contrast, in stage 2 there was no significant difference in the enzymatic infarct size between the three groups (placebo, 100 and 150 mg eniporide). Overall there was no effect of eniporide on clinical outcome of secondary end points. However, a subgroup of patients ($n = 322$, 150 mg eniporide), in whom reperfusion was initiated more than 4 h after symptom onset, showed a significant reduction of heart failure symptoms when compared to the control group.

In the GUARDIAN (*Guard During Ischemia Against Necrosis*) trial ($n = 11\,590$), the cardioprotective efficacy of cariporide was limited to high-risk patients who underwent coronary artery bypass graft (CABG) [67]. This trial failed to document benefit of cariporide over placebo on the primary end point of death or MI assessed after 36 days. Administered to the subpopulation of patients who underwent CABG, cariporide (120 mg three times a day) reduced of 25% the relative risk in the primary end point of death or MI. Further trials are warranted to confirm the cardioprotective benefit of NHE inhibitors in patients undergoing CABG surgery. The recently initiated EXPEDITION (Na^+/H^+ Exchanger Inhibition to Prevent Coronary Events in Acute Cardiac Conditions) trial will test the hypothesis that NHE inhibition results in a reduction of MI in such patients [70].

These mixed results of clinical investigations with eniporide and cariporide contrast with the encouraging results obtained from preclinical studies, and the potential advantage of NHE inhibitors over other therapies claim further trials. Beside the cardioprotective effects of NHE1 inhibitors, the positive preclinical results obtained in the treatment of brain and renal ischemia reperfusion should be also verified in clinical trials [62–66].

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United States Patent [19]

Cherksey

[11] **Patent Number: 4,950,591**

[45] **Date of Patent: Aug. 21, 1990**

[54] **MEMBRANE NA+ CHANNEL PROTEIN AND RELATED THERAPEUTIC COMPOUNDS**

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[21] Appl. No.: **85,462**

[22] Filed: **Aug. 14, 1987**

[51] **Int. Cl.**[5] **G01N 33/53; C07K 3/20**

[52] **U.S. Cl.** **435/7**; 436/63; 436/531; 530/350; 530/395; 530/400; 530/417; 530/813

[58] **Field of Search** 530/350, 395, 400, 417, 530/835, 839, 841, 844, 828, 813; 435/7; 436/63, 531

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[57] **ABSTRACT**

A channel protein has a Na^+/K^+ selectivity of approximately 100 and capable of affecting Na^+ membrane transport. Amiloride derivatives, and amiloride gel materials incorporating such derivatives, are useful in treating membrane transport, cellular volume and cellular pressure disorders and in isolating the channel protein. The channel protein is used in diagnostic assays and screening assays.

7 Claims, 1 Drawing Sheet

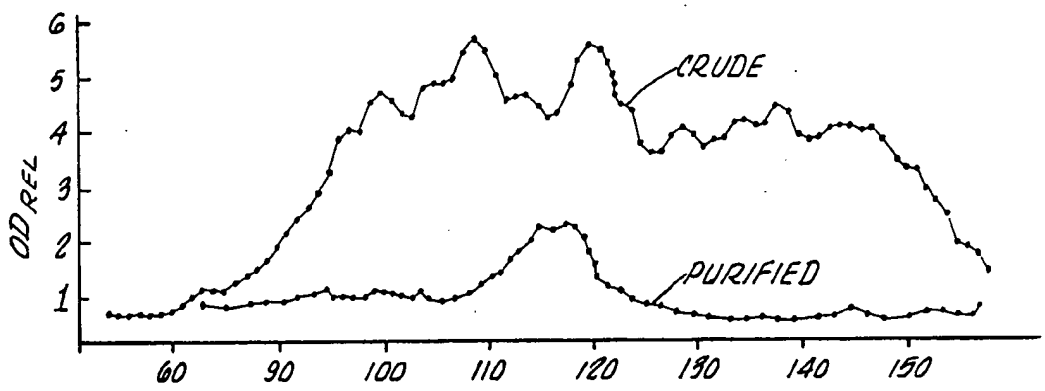
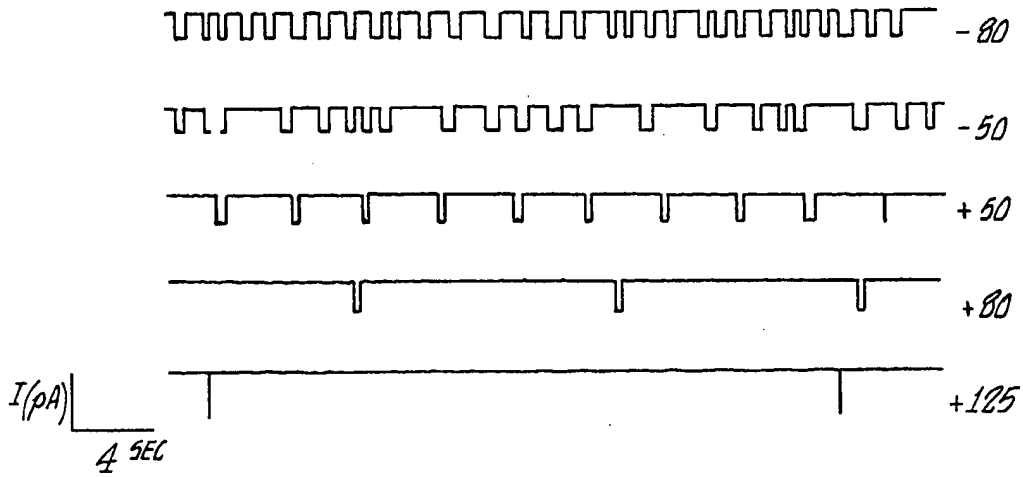


FIG. 1

FIG. 2



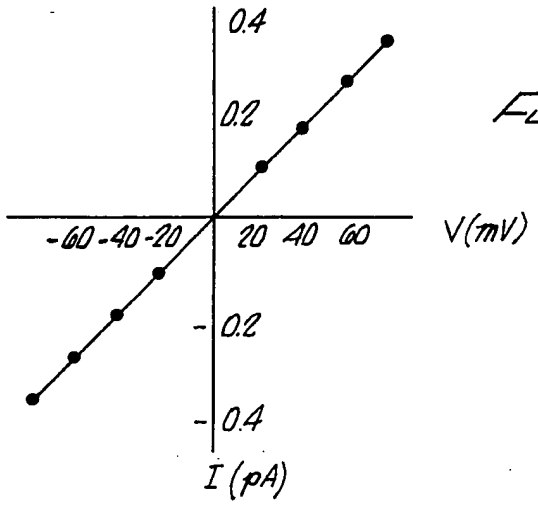


FIG. 3

MEMBRANE NA+ CHANNEL PROTEIN AND RELATED THERAPEUTIC COMPOUNDS

BACKGROUND OF THE INVENTION

The ability to control transport of chemical species across cellular membranes is important from a therapeutic standpoint inasmuch as a number of disease conditions, including glaucoma and certain kidney and stomach disorders, are directly related to such transport. In addition, a better understanding of the mechanisms for such transport can be expected to yield improved therapeutic and diagnostic tools.

It is thought that transport of ions, including Na^+, across membranes is controlled in part by membrane channel proteins which exist in certain membranes and which act to transport or "channel" ions across such membranes. Only about six or so protein channels have actually been isolated. Epithelial Na^+ channel proteins have been discussed by Palmer in *J. Membrane Biol.*, Vol. 96, pp. 97–106 (1987). Agents which selectively block ion transport have been described, as for example amiloride (3,5-diamino-N-(aminoiminomethyl)-6-chloropyrazinecarboxamide). Amiloride is widely thought to interact with a Na^+/H^+ exchanger at high concentrations and a Na^+ channel protein at much lower concentrations. By utilizing the channel protein binding compound amiloride in affinity gels, it has been shown in investigations relating to the present invention to be possible to isolate and purify the Na^+ channel protein. Both the channel protein and the binding compounds and associated affinity gels are useful in therapeutic control of membrane transport or in developing assays related to membrane control.

The full disclosure and claims of applicant's copending application, Ser. No. 948,262, is incorporated herein by reference.

SUMMARY OF THE INVENTION

The present invention involves a newly-isolated membrane channel protein which has been found to be related to Na^+ transport across cellular membranes. The Na^+/K^+ selectivity of the protein is approximately 100. The channel protein has been found in a variety of cell samples including kidney and trachea epithelial cells. Discovery and isolation of this channel protein provides an avenue for regulating ion transport across membranes by utilizing transport blockers which bind to the peptide. Such regulation may also be modulated by the use of Ca^{++}, pH or other media adjustments in in vivo or in vitro systems.

It has also been discovered that the channel protein blocking compound amiloride and derivatives thereof may be used to regulate the transport activity of the channel protein by virtue of their ability to bind to the peptide. Such compounds would be useful in oral, ocular, topical or other administration media to correct membrane transport, cellular volume or cellular pressure disorders such as those associated with glaucoma, gastric ulcers, diuresis problems, hypertension, obesity and the like. Preferred blocking compounds include the reaction products of amiloride and alkanediols, particularly ethane-1,2-diol (ethylene glycol), propane-1,3-diol, butane-1,4-diol, pentane-1,5-diol and the like.

The foregoing channel protein blocking compounds may also be covalently bonded to suitable support media, including monosaccharide or polysaccharide support materials such as those commonly used for gel affinity chromatography, to yield materials which are useful in isolating and purifying the channel protein. Bonding of the amiloride molecule to the support media is achieved in a manner which preserves the ability of the amiloride to bind to the peptide channel, and may be achieved using a glycol or other "linker" group. Such "affinity gel" materials are also useful in their own right as therapeutic compounds capable of regulating membrane transport, cellular volume or cellular pressure disorders. The blocking compounds may also be bonded to other useful groups, such as radiolabels or fluorescent labels.

The channel protein is useful in developing diagnostic assays, as for example immunoassays, relating to membrane transport, cellular volume or cellular pressure disorders in appropriate membrane systems. It may also be used in in vitro screening assays to screen large numbers of test compounds for activity in modulating transport, volume and pressure controls in in vivo and in vitro systems.

Thus, in one respect, the present invention relates to a new and useful protein which has been shown to act as a channel for Na^+ ions in a variety of naturally-occurring cellular membranes. The protein is useful as an agent for developing diagnostic assays, as for example immunoassays utilizing monoclonal or polyclonal antibodies, which may be directed toward determining, in histopathological samples, the extent to which volume and pressure problems may result from abnormally high or abnormally low presence of the Na^+ channel in appropriate membranes. In another aspect, the channel protein is useful as an agent for measuring in vitro the effectiveness of experimental compounds in displacing known blockers of the channel protein, thus furnishing a relatively inexpensive means for screening such compounds for therapeutic transport-modulation activity.

The present invention also provides methods for producing the channel protein. These methods involve the use of new and useful compositions which bind to and/or block the channel protein, including such compositions covalently bonded to appropriate support media including modified polysaccharide support materials derived from, for example, purified agarose, dextran, cellulose or short-chain polysaccharides or monosaccharides such as glucose or dextrose. Such compositions, when bonded to a support structure, are useful in purifying the channel protein from membranal material. In addition, the compositions in their pure and/or support media-bound forms are useful in treating transport-related disorders such as ulcers, diuresis-related disorders, diarrhea, glaucoma, hypertension and obesity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph depicting gel filtration elution curves for the Na^+ channel protein of the present invention, eluted both in crude membranal homogenate and amiloride affinity gel-purified forms.

FIG. 2 is a depiction of typical single-channel recording tracings obtained for the Na^+ channel protein at potentials ranging from -80 to $+125$ mV.

FIG. 3 is a graph representing an i-V curve for the Na^+ channel protein, derived from typical single-channel recording data.

DETAILED DESCRIPTION

The diuretic and antihypertensive drug amiloride is known to block cation transport across membranes.

Derivatives of the drug have been shown in gel affinity chromatography experiments relating to the present invention to be useful in purifying a membrane protein from bovine kidney cortex and bovine trachea epithelia apical membranes. This protein has been shown to be a membranal "channel protein" which incorporates a Na^+ channel, as ascertained from electrical measurements on the purified protein inserted into planar lipid bilayers.

It has been found that glycol adducts of amiloride are particularly useful in the practice of the present invention. Thus, a glycol of from two to six or more carbons may be reacted with amiloride at a pH of about 9 to form a soluble, glycolated amiloride derivative that retains the Na^+ channel protein blocking activity of the native amiloride. The glycol portion of the adduct may be used as a "linker group" to bond the amiloride to other materials in a manner which preserves the amiloride activity.

It will be recognized that derivatives of amiloride which exhibit the ability of the native compound to block the Na^+ channel protein will also be useful in the practice of the present invention, and may also be used to form the, e.g., glycol adduct compositions and affinity gel preparations described herein. Other derivatizing groups capable of reacting with amiloride in a manner which retains the molecule's biological activity while affording, for example, increased solubility or specifically reactive functional groups will also be useful in the practice of the present invention.

EXAMPLE 1

Preparation of Glycol Adduct of Amiloride

By way of illustrative example, 1 g of amiloride is dissolved in 50 ml of ethylene glycol. The pH is adjusted by addition of sufficient 1 N sodium hydroxide solution to maintain a pH of 9 for 5 hours. The pH is then changed to pH 5 by addition of dilute HCl. The adduct of amiloride with ethylene glycol is isolated by salting, extraction, washing and further purification if needed.

In like manner, but substituting propane-1,3-diol and other alkanediols of the formula $HO—(CH_2)_n—OH$ and $HO—C_nH_{2n}—OH$, where n is 3, 4, 5 or greater, there are produced the propylene glycol adduct and other alkanediol adducts of amiloride.

A preferred amiloride derivative is the ethylene glycol adduct of amiloride. This derivative, confirmed by NMR analysis, retains biological activity similar to that of the unmodified molecule. This and other adducts of amiloride may be linked to, for example, affinity gel support materials to form amiloride "affinity gels" having channel protein-binding activity. The discovery of such amiloride adduct derivatives and uses therefor are particularly important aspects of the present invention inasmuch as amiloride itself cannot be directly linked to an affinity gel or other support without loss of activity of the drug.

In addition, by virtue of their ability to bind to and block the transport activity of the channel protein, the above adducts, as well as amiloride derivatives and related derivative adducts may also be useful in directly treating disorders related to abnormal transport activity in membranes. For example, the materials are conceived to have useful diuretic activity when given orally and to be useful when applied topically for the prevention and treatment of glaucoma. In these uses, the materials may be formulated in vehicles standard for oral or ocular use and are given in amounts sufficient to affect aqueous transport but below amounts having toxic or irritating properties.

The amiloride affinity gels used in purifying the present Na^+ channel protein may be formed by covalently bonding the above amiloride ethylene glycol adduct or analogous amiloride adducts to a modified polysaccharide support, as for example a support derived from a purified agarose (e.g, Sepharose ® (Pharmacia)), from an alpha-linked dextran (e.g., Sephadex ® (Pharmacia)) or from cellulose. Short-chain saccharides, as for example glucose or dextrose, may also be used. Although such affinity purification compositions will be referred to herein as "affinity gels," when the polysaccharide is short or is not cross-linked the composition may not in fact be a typical gel.

Preparation of amiloride gels may be accomplished using a glycol linker group and CNBr by methods standard in the art. A preferred method involves the use of an ammonium salt, as for example NH_4Cl or $(NH_4)_2SO_4$, to generate free ammonia species which derivatize the gel and allow rapid formation of reactive coupling species.

EXAMPLE 2

Preparation of Amiloride Affinity Gel

By way of illustrative example, 100 ml of agarose beads are washed with water, freed of interstitial water by suction filtration, and then added to 80 ml of water. To the suspension is then added an ammonium salt (for example, NH_4Cl or $(NH_4)_2SO_4$) to a concentration of approximately 1 M. The pH is then increased to approximately 9, causing generation of "free ammonia" species. After about five minutes, a glycol adduct of amiloride, as for example, the ethylene glycol adduct, is added while maintaining the pH constant at 9 with NaOH. Reaction is continued until the pH remains constant. The mixture is then brought to a pH of about 4.5 and reduced with, e.g., sodium borohydride at 4° C. for 12 hours. The resulting amiloride gel is washed thoroughly to remove all reducing agent and dried by vacuum filtration. If desired, it may be further dried to a powder by drying under vacuum at 30°–60° C. The resultant gel or powder is useful as reagent to isolate the purified Na^+ channel. It is also useful when administered orally in producing diuresis, inhibiting gastric acid secretion and treating diarrhea. It is useful when applied topically in treating glaucoma.

EXAMPLE 3

Preparation of Amiloride Affinity Gel Using CNBr

Using procedures described in *Affinity Chromatography* by W. H. Scouten, pages 45–49 (John Wiley and Sons, 1981) and in *Affinity Chromatography* edited by P. D. G. Dean, W. S. Johnson and F. A. Middle (IRL Press, 1985), gels may also be produced from the ethylene glycol and other glycol adducts of amiloride and various polysaccharide gels, such as Sepharose ®, Sephadex ® and cellulose. In one such procedure, the support (Sepharose ® 4B) is first reacted with ethylene glycol, using CNBr, to provide a spacer group. The glycol-Sepharose ® is then reacted with amiloride at pH 9 for 5 hours. The pH is then lowered to 4.5 and sodium borohydride is added. The mixture is allowed to stir overnight at 4° C. The amiloride-Sepharose ® is then washed extensively with Tris—HCl, Tris base,

sodium bicarbonate and then water. The final product has a distinctive yellow color.

In the above examples, ethylene glycol may be replaced by other glycols, such as HO—$(CH_2)_n$—OH and HO—C_nH_{2n}—OH where n is a integer of from 3 to about 11, to afford the correspondingly-modified amiloride gels in which the linker adduct contains, for example, 3, 4, 5 or more carbon atoms.

In addition, agarose may be replaced by commercially available supports such as an alpha-linked dextran (e.g., Sephadex ®), cellulose, simple sugars or complex carbohydrates to afford the corresponding gels containing the modified amiloride. Similar gels may be formed using short-chain polysaccharides or monosaccharides such as glucose or dextrose using the above methods or other methods standard in the art.

It will be noted that appropriate gels may be formed by reaction of a preformed amiloride adduct derivative with a gel material, by reaction of amiloride with a suitably prepared (i.e., linker group-primed) gel, or by simultaneous in situ reaction of amiloride, linker group and gel. Specific conditions and modifications to these procedures will also be recognized by those skilled in the art.

In addition, it will be apparent that support media other than materials commonly used for affinity gel purposes, as well as amiloride derivatives other than glycol adducts, will be useful in the practice of the present invention. For example, therapeutic benefits such as reduction of intraocular pressure attributable to amiloride may be enhanced by utilizing the present invention to bond amiloride to materials or functional groups which increase intraocular absorption or which lengthen intraocular residence time.

The above amiloride gel materials are useful in the isolation and purification of the Na^+ channel peptide and may also be used directly as therapeutic agents. The gel materials may be administered orally to human or animal subjects to inhibit gastric acid secretion and thus to treat or prevent gastric ulcer, to enhance diuresis and to prevent diarrhea, particularly diarrhea associated with cholera. Furthermore, such gel materials, especially when derived from short-chain saccharides such as glucose or dextrose, or from dextran, may be applied topically to the eye to reduce intraocular pressure, as for example to treat glaucoma.

The Na^+ channel peptide may be isolated and purified using the amiloride affinity gels described above following methods well known in the art, as discussed below.

EXAMPLE 4

Purification of Channel Peptide

By way of illustrative example, bovine kidney cortex cell membranes or bovine trachea apical cell membranes are collected and pooled until the pooled membranes contain about 100 mg of protein as determined by the Lowry method. The pooled membranes in 100 mM HEPES, pH 7.4, are centrifuged at 40,000 g for 30 minutes. The pelleted material was resuspended in 100 mM HEPES, pH 7.4, containing 3% sodium cholate (4.5–5.5 mg of membrane protein/3 ml of solution) containing in addition bacitracin (100 microgram/g), phenylmethylsulfonyl fluoride (0.5%) and iodoacetamide (1 mM). The mixture is chilled on ice for 60 minutes with occasional agitation, then centrifuged at 40,000 g for 30 minutes. The supernatant, containing the solubilized protein, may be purified immediately or frozen at −70° for later purification.

The solubilized protein preparation obtained from about 10 g of cell membrane is purified using 100 ml of the affinity gel described in Example 2. The protein solution and gel are stirred overnight in the cold. The gel is washed with 20 volumes of HEPES solution, pH 7.2, by filtration. The adsorbed protein is eluted with 10^{-3} M amiloride in dilute saline containing 0.5% cholate by stirring for two hours. The elution was repeated and the combined eluates are dialyzed against 100 mM HEPES/1% sodium cholate for one week in the cold, changing buffers once or twice a day. The residue is reapplied to the affinity gel and the batch purification process repeated. The resulting purified protein is then resubmitted to analysis and characterization. Typically, 10 g of cell membrane yields about 0.1 microgram of purified protein.

The purified protein was analyzed by gel filtration chromatography (Sepharose ® CL-4B) and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). FIG. 1 shows a typical elution curve from a Sepharose ® 4B column of crude tracheal membrane homogenate in 400 mM sucrose (top) and protein purified by the amiloride affinity gel (lower). Elution from the column (1.5×155 cm, i.d.) was at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected and protein was determined as the optical absorbance at 280 nm (A_{280}).

The unexpectedly high absorbance for the protein peak appears to be due to bound detergent. Such detergent would act to "amplify" the elution signal, allowing measurement of smaller amounts of protein than would be possible otherwise. However, the bound detergent also interferes with the accurate determination of molecular weight. Present data suggests that the molecular weight of the isolated channel protein is approximately 200–220 kD.

Typical channel recordings obtained at potentials ranging from −80 to +125 mV in a reconstituted lipid bilayer system are shown in FIG. 2. At the most positive potentials (equivalent to negative cell potentials under the electrophysiological convention), the channel was essentially closed with an occasional short opening seen sporadically. With depolarization, the channel was seen to be open with increasing probability. However, even at highly depolarized potentials (−80 mV), the channel exhibited frequent closings. At potentials greater than −150 mV (physiologically unrealistic), the channel was found to be essentially in the open state. Further studies appear necessary to characterize the opening probabilities and gating potentials in a definitive manner.

The single-channel data typified by FIG. 2 yields the i-V curve in FIG. 3, which shows a conductance of 5–7 pS, somewhat higher than that found for the channel protein from crude membrane preparations.

Under bi-ionic conditions, the Na^+/K^+ selectivity ratio of the channel protein was greater than 100. The isolated channel exhibited half-saturation at about 90 mM Na^+.

The effect of amiloride on sodium concentration has been studied. The purified channel was found to be extremely amiloride sensitive, with an IC_{50} of about 0.07 micromolar.

In both the crude membrane preparation and in purified channel preparations, a pH/Ca^{++} effect was apparent in that activity was not seen in unbuffered, acidic solutions. Buffering to pH 7.2 elicited the channel activ-

ity. While this effect, in the main, is most likely due to a pH effect, the concomitant changes in free Ca++ may also contribute.

EXAMPLE 5

Transport Electrical Properties of the Protein Channel

The electrical properties of the purified protein were studied by first chromatographing it on Sephadex® G-150 and then reconstituting the void volume into lipid bilayers (phosphatidylethanolamine/phosphatidylcholine=4/1) formed by sonication dialysis (Coronado, et al., *Biophys. J.*, Vol. 43, pp. 231-236 (1983)). The microelectrode (i.d.=1 micrometer) and the external bath contained 0.5 M sodium citrate, Na sodium gluconate or potassium gluconate. All contained 1 mM $CaCl_2$ and 5 mM Tris, pH 7.4. Amiloride could be included. The currents were discrete and fluctuating with a unitary-channel conductance of 6 pS.

Typical channel recordings obtained at potentials ranging from −80 to +125 mV are shown in FIG. 2.

The data obtained for trachea epithelial preparations is summarized in Table I, which shows results for both the purified reconstituted channel protein and for the crude membrane preparation.

TABLE I

COMPARISON OF CHANNEL PROPERTIES		
	PURIFIED	MEMBRANE
Conductance (pS)	5-7	2-3
Saturation (mM)	90	85
Na^+ selectivity	100	100
Amiloride, IC_{50} (micromolar)	.07	.1
pH/Ca^{++}	+	+

The results which have been obtained for the isolated purified airway epithelium sodium channel compare favorably with those reported for Na^+ channels in the natural state in other tissues. Palmer, L., *J. Membrane Biol.*, Vol. 96, pp. 97-106 (1987). In particular, both the channel conductance and its selectivity suggest its identification as the epithelial Na^+ channel. This conclusion is strengthened by the protein's high sensitivity to amiloride.

The Na^+ peptide channel disclosed herein may be used in methods now standard in the art to produce polyclonal or monoclonal antibodies thereto. These antibodies may be used in accordance with standard immunofluorescence or radioimmunoassay techniques to produce diagnostic assays capable of determining, in histopathological samples, the extent to which volume and pressure problems may result from abnormally high or abnormally low presence of the peptide channel in appropriate membranes. Alternately, radio-labeled or fluorescent-labeled derivatives of amiloride, as for example derivatives labeled on ligand adducts such as the glycol adducts described herein, may be used to quantitate the presence of the channel protein.

The purified Na^+ channel may also be used in conjunction with, for example, radio-labeled amiloride in a screening procedure capable of evaluating the ability of new chemical entities to displace the amiloride. Entities that displace the bound labeled channel blocker may be presumed to affect volume and pressure control mechanisms in humans and other animals and are therefore candidates for further in vivo evaluation. The present Na^+ channel peptide is thus useful in providing an in vitro assay methodology capable of screening large numbers of test compounds without requiring the use of large numbers of experimental animals. The methods to be employed in such screening assays are well known to those skilled in the art given the present disclosure.

Given the disclosure of the present invention, it will be apparent to those skilled in the art that various modifications and equivalents to the embodiments described herein will be possible. It is not intended that the scope of the present invention be limited except as by the appended claims.

What is claimed is:

1. A method for screening a chemical entity for activity in modulating membrane transport, cellular volume or cellular pressure, said method including the step of measuring the ability of said chemical entity to bind to a channel protein, wherein said channel protein is a Na^+ channel protein produced by extracting said channel protein from cell membrane material with an affinity gel, said affinity gel comprising amiloride or a derivative of amiloride bonded to a support material.

2. A method for screening a chemical entity for activity in modulating membrane transport, cellular volume or cellular pressure, said method including the step of measuring the ability of said chemical entity to displace amiloride or a derivative of amiloride bound to a channel protein, wherein said channel protein is a Na^+ channel protein produced by extracting said channel protein from cell membrane material with an affinity gel, said affinity gel comprising amiloride or a derivative of amiloride bonded to support material.

3. The method of claim 2 wherein said derivative of amiloride is a radio-labeled or fluorescent-labeled derivative of amiloride.

4. A process for isolating a Na^+ channel protein including the step of extracting said channel protein from cell membrane material with an affinity gel, said affinity gel comprising amiloride or a derivative of amiloride bonded to a support material.

5. The process of claim 4 wherein said support material is a polysaccharide or a monosaccharide support material.

6. The process of claim 4 wherein said amiloride or derivative of amiloride is bonded to said support material with a linker group formed from an alkanediol of the formula $HO—(CH_2)_n—OH$ or $HO—C_nH_{2n}—OH$, wherein n is an integer of from 2 to 11.

7. The process of claim 6 wherein said alkanediol is ethylene glycol.

* * * * *

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(3)

Refer to the general discussion of these products beginning on page 2281

Beta-Adrenergic Blocking Agents

Actions:

Pharmacology: Timolol, levobunolol, carteolol and metipranolol are noncardioselective (β_1 and β_2) β-blockers; betaxolol is a cardioselective (β_1) β-blocker. Topical β-blockers do not have significant membrane-stabilizing (local anesthetic) actions or intrinsic sympathomimetic activity. They reduce elevated and normal intraocular pressure (IOP), with or without glaucoma.

The exact mechanism of ocular antihypertensive action is not established, but it appears to be a reduction of aqueous production. However, some studies show a slight increase in outflow facility with timolol and metipranolol.

These agents reduce IOP with little or no effect on pupil size or accommodation. Blurred vision and night blindness often associated with miotics are not associated with these agents. In addition, in patients with cataracts, the inability to see around lenticular opacities when the pupil is constricted is avoided. These agents may be absorbed systemically (see Warnings).

Pharmacokinetics:

Pharmacokinetics of Ophthalmic β-Adrenergic Blocking Agents

Drug	β-receptor selectivity	Onset (min)	Maximum effect (hr)	Duration (hr)
Carteolol	β_1 and β_2	nd	nd	12
Betaxolol	β_1	30	2	12
Levobunolol	β_1 and β_2	< 60	2 to 6	12 to 24
Metipranolol	β_1 and β_2	≤ 30	≈ 2	12 to 24
Timolol	β_1 and β_2	30	1 to 2	12 to 24

nd = No data

Clinical pharmacology: Timolol – In controlled studies of untreated IOP of ≥ 22 mm Hg, timolol 0.25% or 0.5% twice daily caused greater IOP reduction than 4% pilocarpine solution 4 times daily or 2% epinephrine HCl solution twice daily. In comparative studies, mean IOP reduction was 31% to 33% with timolol, 22% with pilocarpine and 28% with epinephrine.

In ocular hypertension, effects of timolol and acetazolamide are additive. Timolol, generally well tolerated, produces fewer and less severe side effects than pilocarpine or epinephrine. Timolol has been well tolerated in patients wearing conventional (PMMA) hard contact lenses.

Betaxolol ophthalmic was compared to ophthalmic timolol and placebo in patients with reactive airway disease. Betaxolol had no significant effect on pulmonary function as measured by Forced Expiratory Volume (FEV_1), Forced Vital Capacity (FVC) and FEV_1/VC. Also, action of isoproterenol was not inhibited. Timolol significantly decreased these pulmonary functions. No evidence of cardiovascular β-blockade during exercise was observed with betaxolol. Mean arterial blood pressure was not affected by any treatment; however, timolol significantly decreased mean heart rate. Betaxolol reduces mean IOP 25% from baseline. In controlled studies, the magnitude and duration of the ocular hypotensive effects of betaxolol and timolol were clinically equivalent.

Clinical observation of glaucoma patients treated with betaxolol solution for up to 3 years shows that the IOP-lowering effect is well maintained.

Betaxolol has been successfully used in glaucoma patients who have undergone laser trabeculoplasty and have needed long-term antihypertensive therapy. The drug is well tolerated in glaucoma patients with hard or soft contact lenses and in aphakic patients.

Levobunolol effectively reduced IOP in controlled clinical studies from 3 months to over 1 year when given topically twice daily; IOP was well maintained. The mean IOP decrease from baseline was between 6.8 and 9 mm Hg with 0.5% levobunolol.

Metipranolol reduced the average intraocular pressure approximately 20% to 26% in controlled studies of patients with IOP > 24 mm Hg at baseline. Clinical studies in patients with glaucoma treated for up to 2 years indicate that an intraocular pressure lowering effect is maintained.

Carteolol produced a median percent IOP reduction of 22% to 25% when given twice daily in clinical trials ranging from 1.5 to 3 months.

Indications:

Lowering IOP in patients with chronic open-angle glaucoma.

For specific approved indications, refer to individual drug monographs.

(Continued on following page)

Contraindications:

Bronchial asthma, a history of bronchial asthma or severe chronic obstructive pulmonary disease; sinus bradycardia; second-degree and third-degree AV block; cardiac failure; cardiogenic shock; hypersensitivity to any component of the products.

Warnings:

Systemic absorption: These agents may be absorbed systemically. The same adverse reactions found with systemic β-blockers (see group monograph in Cardiovascular section) may occur with topical use. For example, severe respiratory reactions and cardiac reactions, including death due to bronchospasm in asthmatics, and rarely, death associated with cardiac failure, have been reported with topical β-blockers. Levobunolol and metipranolol may decrease heart rate and blood pressure, and betaxolol has had adverse effects on pulmonary and cardiovascular parameters. Detectable, perhaps significant serum timolol levels may be achieved in some patients. Exercise caution with all of these agents.

Cardiovascular: Timolol can decrease resting and maximal exercise heart rate even in normal subjects.

Cardiac failure – Sympathetic stimulation may be essential for circulation support in diminished myocardial contractility; its inhibition by β-receptor blockade may precipitate more severe failure.

In patients without history of cardiac failure, continued depression of myocardium with β-blockers may lead to cardiac failure. Discontinue at the first sign or symptom of cardiac failure.

Non-allergic bronchospasm patients, or patients with a history of chronic bronchitis, emphysema, etc, should receive β-blockers with caution; they may block bronchodilation produced by catecholamine stimulation of β_2-receptors.

Major surgery: Withdrawing β-blockers before major surgery is controversial. Beta-receptor blockade impairs the heart's ability to respond to β-adrenergically mediated reflex stimuli. This may augment the risk of general anesthesia. Some patients on β-blockers have had protracted severe hypotension during anesthesia. Difficulty restarting and maintaining heartbeat has been reported. In elective surgery, gradual withdrawal of β-blockers may be appropriate.

The effects of β-blocking agents may be reversed by β-agonists such as isoproterenol, dopamine, dobutamine or norepinephrine.

Diabetes mellitus: Administer with caution to patients subject to spontaneous hypoglycemia or to diabetic patients (especially labile diabetics). Beta-blocking agents may mask signs and symptoms of acute hypoglycemia.

Thyroid: Beta-adrenergic blocking agents may mask clinical signs of hyperthyroidism (eg, tachycardia). Manage patients suspected of developing thyrotoxicosis carefully to avoid abrupt withdrawal of β-blockers which might precipitate thyroid storm.

Cerebrovascular insufficiency: Because of potential effects of β-blockers on blood pressure and pulse, use with caution in patients with cerebrovascular insufficiency. If signs or symptoms suggesting reduced cerebral blood flow develop, consider alternative therapy.

Carcinogenesis: In mice receiving oral metipranolol doses of 5, 50 and 100 mg/kg/day, females receiving the low dose had an increased number of pulmonary adenomas.

Pregnancy: Category C. There are no adequate and well controlled studies in pregnant women. Use during pregnancy only if the potential benefits outweigh potential hazards to the fetus.

Carteolol – Increased resorptions and decreased fetal weights occurred in rabbits and rats at maternally toxic oral doses ≈ 1052 and 5264 times the maximum human dose, respectively. A dose-related increase in wavy ribs was noted in the developing rat fetus when pregnant rats received oral daily doses ≈ 212 times the maximum human dose.

Betaxolol – In oral studies with rats and rabbits, evidence of post-implantation loss was seen at dose levels above 12 mg/kg and 128 mg/kg, respectively. Betaxolol was not teratogenic, however, and there were no other adverse effects on reproduction at subtoxic dose levels.

Levobunolol – Fetotoxicity was observed in rabbits at doses 200 and 700 times the glaucoma dose.

Metipranolol – Increased fetal resorption, fetal death and delayed development occurred in rabbits receiving 50 mg/kg orally during organogenesis.

Timolol – Doses 1000 times the maximum recommended human oral dose were maternotoxic in mice and resulted in increased fetal resorptions. Increased fetal resorptions were seen in rabbits at 100 times the maximum recommended human oral dose.

(Warnings continued on following page)

Beta-Adrenergic Blocking Agents (Cont.)

Warnings (Cont.):

Lactation: It is not known whether betaxolol, levobunolol or metipranolol are excreted in breast milk. Systemic β-blockers and topical timolol maleate are excreted in milk. Carteolol is excreted in breast milk of animals. Exercise caution when administering to a nursing mother.

Because of the potential for serious adverse reactions from timolol in nursing infants, decide whether to discontinue nursing or discontinue the drug. Ophthalmic timolol that is absorbed appears to concentrate in breast milk approximately sixfold.

Children: Safety and efficacy for use in children have not been established.

Precautions:

Angle-closure glaucoma: The immediate objective is to reopen the angle, requiring constriction of the pupil with a miotic. These agents have little or no effect on the pupil. When they are used to reduce elevated IOP in angle-closure glaucoma, use with a miotic.

Muscle weakness: Beta-blockade may potentiate muscle weakness consistent with certain myasthenic symptoms (eg, diplopia, ptosis, generalized weakness). Timolol has increased muscle weakness in some patients with myasthenic symptoms.

Long-term therapy: Diminished responsiveness to betaxolol and timolol after prolonged therapy has been reported. However, in long-term studies (2 and 3 years), no significant differences in mean IOP were observed after initial stabilization.

Sulfite sensitivity: Some of these products contain sulfites which may cause allergic-type reactions (eg, hives, itching, wheezing, anaphylaxis) in certain susceptible persons. Although the overall prevalence of sulfite sensitivity in the general population is probably low, it is seen more frequently in asthmatics or in atopic nonasthmatic persons.

Drug Interactions:

Ophthalmic Beta Blocker Drug Interactions

Precipitant drug	Object drug*		Description
Beta blockers, ophthalmic	Beta blockers, oral	↑	Use topical β-blockers with caution because of the potential for additive effects on systemic β-blockade.
Beta blockers, ophthalmic	Epinephrine, ophthalmic	↔	The use of epinephrine with topical β-blockers is controversial. Some reports indicate the initial effectiveness of the combination decreases over time. In one case verified by rechallenge, combined use of topical epinephrine and topical timolol appeared to result in hypertension resulting from unopposed α-adrenergic stimulation. However, this combination has been used to reduce IOP.
Beta blockers, ophthalmic	Quinidine	↑	One case of sinus bradycardia has been reported with the coadministration of ophthalmic timolol. The incidence was reaffirmed by a negative rechallenge with the β-blockers alone and positive rechallenge with the combination.
Beta blockers, ophthalmic	Verapamil	↑	Coadministration of ophthalmic timolol has caused bradycardia and asystole.

* ↑ = Object drug increased ↔ = Undetermined effect

Other drugs that may interact with the systemic β-adrenergic blocking agents may also interact with the ophthalmic agents. For further information refer to the β-blocker group monograph in the Cardiovascular section.

Adverse Reactions:

The following adverse reactions have occurred with ophthalmic use of the β_1 and β_2 (nonselective) adrenergic blocking agents:

Systemic:

CNS – Headache; depression.

Cardiovascular – Arrhythmia; syncope; heart block; cerebral vascular accident; cerebral ischemia; congestive heart failure; palpitation.

Digestive – Nausea.

Skin – Hypersensitivity, including localized and generalized rash.

Respiratory – Bronchospasm (predominantly in patients with preexisting bronchospastic disease); respiratory failure.

Endocrine – Masked symptoms of hypoglycemia in insulin-dependent diabetics (see Warnings).

Ophthalmic – Keratitis; blepharoptosis; visual disturbances including refractive changes (due to withdrawal of miotic therapy in some cases); diplopia; ptosis.

(Adverse Reactions continued on following page)

Adverse Reactions (Cont.)

The following adverse reactions have occurred with each individual agent:

Carteolol: Ophthalmic – Transient eye irritation, burning, tearing, conjunctival hyperemia, edema (≈25%); blurred/cloudy vision; photophobia; decreased night vision; ptosis; blepharoconjunctivitis; abnormal corneal staining; corneal sensitivity.

Systemic – Bradycardia; decreased blood pressure; arrhythmia; heart palpitation; dyspnea; asthenia; headache; dizziness; insomnia; sinusitis; taste perversion.

Betaxolol: Ophthalmic – Brief discomfort (> 25%); occasional tearing (5%). Rare: Decreased corneal sensitivity; erythema; itching; corneal punctate staining; keratitis; anisocoria; photophobia.

Systemic – Insomnia; depressive neurosis (rare).

Metipranolol: Ophthalmic – Transient local discomfort; conjunctivitis; eyelid dermatitis; blepharitis; blurred vision; tearing; browache; abnormal vision; photophobia; edema.

Systemic – Allergic reaction; headache; asthenia; hypertension; myocardial infarction; atrial fibrillation; angina; palpitation; bradycardia; nausea; rhinitis; dyspnea; epistaxis; bronchitis; coughing; dizziness; anxiety; depression; somnolence; nervousness; arthritis; myalgia; rash.

Levobunolol: Ophthalmic – Transient burning/stinging (25%); blepharoconjunctivitis (5%); iridocyclitis (rare); decreased corneal sensitivity.

Cardiovascular effects may resemble timolol.

CNS – *Rare:* Ataxia; dizziness; lethargy.

Dermatologic – *Rare:* Urticaria; pruritis.

Timolol: Ophthalmic – Ocular irritation including conjunctivitis; blepharitis; keratitis; blepharoptosis; decreased corneal sensitivity; visual disturbances including refractive changes (due, in some cases, to withdrawal of miotics); diplopia; ptosis.

CNS – Headache; dizziness; depression; fatigue; lethargy; hallucinations; confusion.

Cardiovascular – Bradycardia; arrhythmia; hypotension; syncope; heart block; cerebral vascular accident; cerebral ischemia; congestive heart failure; palpitation; cardiac arrest. These generally occur in the elderly or those with preexisting cardiovascular problems.

Respiratory – Bronchospasm (mainly in patients with preexisting bronchospastic disease); respiratory failure; dyspnea.

Other – Aggravation of myasthenia gravis; alopecia; nail pigmentary changes; nausea; hypersensitivity including localized and generalized rash; urticaria; asthenia; sexual dysfunction including impotence, decreased libido and decreased ejaculation; hyperkalemia; masked symptoms of hypoglycemia in insulin-dependent diabetics; diarrhea; paresthesia.

Causal relationship unknown – Hypertension; chest pain; dyspepsia; anorexia; dry mouth; behavioral changes including anxiety, disorientation, nervousness, somnolence and other psychic disturbance; aphakic cystoid macular edema; retroperitoneal fibrosis.

Systemic β-adrenergic blocker-associated reactions: Consider potential effects with ophthalmic use. See Warnings.

Overdosage:

If ocular overdosage occurs, flush eye(s) with water or normal saline. If accidentally ingested, efforts to decrease further absorption may be appropriate (gastric lavage).

The most common signs and symptoms of overdosage from systemic β-blockers are bradycardia, hypotension, bronchospasm and acute cardiac failure. If these occur, discontinue therapy and initiate appropriate supportive therapy. See group monograph in Cardiovascular section.

Patient Information:

Refer to page 2278 for more complete information.

Transient stinging/discomfort is relatively common with these agents. Notify physician if severe.

Administration:

Monitoring: The IOP-lowering response to betaxolol and timolol may require a few weeks to stabilize. Determine the IOP during the first month of treatment. Thereafter, determine IOP on an individual basis.

Because of diurnal IOP variations in individual patients, satisfactory response to twice a day therapy is best determined by measuring IOP at different times during the day. Intraocular pressures ≤ 22 mm Hg may not be optimal to control glaucoma in each patient; therefore, individualize therapy.

Concomitant therapy: If IOP is inadequately controlled with these agents, institute concomitant pilocarpine, other miotics, dipivefrin or systemic carbonic anhydrase inhibitors.

The use of epinephrine in combination with topical β-blockers is controversial. Some reports indicate the initial effectiveness of the combination decreases over time (see Drug Interactions).

(Products listed on following pages)

METIPRANO
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[3] With 0.005%
[4] With 1.4% pol

Beta-Adrenergic Blocking Agents (Cont.)

METIPRANOLOL HCl

Indications:
Treatment of ocular conditions in which lowering intraocular pressure is likely to be of therapeutic benefit, including patients with ocular hypertension and in patients with chronic open angle glaucoma.

Administration and Dosage:
Usual dose: One drop in the affected eye(s) twice a day. If the patient's IOP is not at a satisfactory level on this regimen, more frequent administration or a larger dose is not known to be of benefit. Concomitant therapy to lower IOP can be instituted. C.I.*

Rx	OptiPranolol (Bausch & Lomb)	Solution: 0.3%	In 5 or 10 ml dropper bottles.[1]	1.3

CARTEOLOL HCl

Indications:
Treatment of chronic open-angle glaucoma and intraocular hypertension. It may be used alone or in combination with other intraocular pressure lowering drugs.

Administration and Dosage:
Usual dose: One drop in affected eye(s) twice daily. If the patient's IOP is not at a satisfactory level on this regimen, concomitant therapy can be instituted. C.I.*

Rx	Ocupress (Otsuka America[2])	Solution: 1%	In 5 and 10 ml dropper bottles.[3]	86

LEVOBUNOLOL HCl

Indications:
Lowering IOP in chronic open-angle glaucoma or ocular hypertension.

Administration and Dosage:
Usual dose: 1 drop in the affected eye(s) once or twice a day. C.I.*

Rx	Betagan Liquifilm (Allergan)	Solution: 0.25%	In 5 and 10 ml dropper bottles with B.I.D. *C Cap.*[4]	NA
		0.5%	In 2 ml dropper bottle[4] and 5, 10 and 15 ml dropper bottles[1] with B.I.D. and Q.D. *C Cap.*[4]	2.2

* Cost Index based on cost per ml.
[1] With 0.004% benzalkonium chloride and EDTA.
[2] Otsuka America Pharmaceutical, Inc., 1201 Third Avenue, Suite 5300, Seattle, WA 98101; 206-682-5300.
[3] With 0.005% benzalkonium chloride.
[4] With 1.4% polyvinyl alcohol, 0.004% benzalkonium chloride, sodium metabisulfite and EDTA.

nplete prescribing information for these products begins on page 2286.

Beta-Adrenergic Blocking Agents (Cont.)

TAXOLOL HCl

ndications:
Treatment of ocular hypertension and chronic open-angle glaucoma. Betaxolol may be used alone or in combination with other antiglaucoma drugs.

Administration and Dosage:
Usual dose: One drop twice daily.
Replacement therapy (single agent): Continue the agent already used and add 1 drop of betaxolol twice daily. The following day, discontinue the previous agent and continue betaxolol. Monitor with tonometry.
Replacement therapy (multiple agents): When transferring from several concomitant antiglaucoma agents, individualize dosage. Adjust 1 agent at a time at intervals of not less than 1 week. One approach is to continue the agents being used and add 1 drop of betaxolol twice daily. The next day, discontinue one of the other agents. Decrease or discontinue the remaining antiglaucoma agents according to patient response.

			C.I.*
Betoptic (Alcon)	Solution: 5.6 mg (equiv. to 5 mg base) per ml (0.5%)	In 2.5, 5, 10 and 15 ml Drop-Tainer bottles.[1]	2.4
Betoptic S (Alcon)	Suspension: 2.8 mg (equiv. to 2.5 mg base) per ml (0.25%)	In 2.5, 5, 10 and 15 ml Drop-Tainer bottles.[1]	2.2

MOLOL MALEATE

Indications:
Effective in lowering IOP in patients with chronic open-angle glaucoma, aphakic patients with glaucoma, some patients with secondary glaucoma and patients with elevated IOP who require lowering of the ocular pressure.
In patients who respond inadequately to multiple antiglaucoma drug therapy, the addition of timolol may produce a further reduction of IOP.

Administration and Dosage:
Initial therapy: 1 drop of 0.25% twice a day. If clinical response is not adequate, change the dosage to 1 drop of 0.5% solution twice a day. If the IOP is maintained at satisfactory levels, change the dosage to 1 drop once a day.
Replacement therapy (single agent): When a patient is transferred from another topical ophthalmic β blocking agent, discontinue that agent after proper dosing on one day, and start treatment on the following day with 1 drop of 0.25% timolol. Increase the dose to 1 drop of 0.5% solution twice a day if clinical response is not adequate.
When changing from another antiglaucoma agent, other than a topical ophthalmic β-blocking agent, on the first day continue with the agent being used and add 1 drop of 0.25% timolol twice daily. The next day, discontinue the previously used agent completely and continue with timolol. If a higher dosage is required, substitute 1 drop of 0.5% twice daily.
Replacement therapy (multiple agents): When transferring from several concomitantly administered agents, individualize dosage. If any of the agents is an ophthalmic β-blocker, discontinue it before starting timolol. Adjust 1 agent at a time and at intervals of not less than 1 week. Continue the agents being used and add 1 drop of 0.25% twice a day. The next day, discontinue 1 of the other antiglaucoma agents. The remaining agents may be decreased or discontinued according to patient response. If a higher dosage is required, substitute 1 drop of 0.5% twice a day.

				C.I.*
Rx	Timoptic (MSD)	Solution: 0.25%	In 2.5, 5, 10 and 15 ml Ocumeter bottles.[2]	2.2
		0.5%	In 2.5, 5, 10 and 15 ml Ocumeter bottles.[2]	2.6
Rx	Timoptic in Ocudose (MSD)	Solution: 0.25%	Preservative free. In UD 60s.	1.3
		0.5%	Preservative free. In UD 60s.	1.5

* Cost Index based on cost per ml.
[1] With 0.01% benzalkonium chloride and EDTA.
[2] With 0.01% benzalkonium chloride.

Refer to the Agents for Glaucoma introductio

Miotics,

Actions:
Pharmacology: The direct-acting miotics a the muscarinic effects of ac tylcholine, cally; these drugs produce pupillary con increase aqueous humor outflow facilit sphincter, causes increased tension on opening of the trabecular meshwork sp flow facility, there is a decrease in intra

Pharmacokinetics:

Miotic	Intraocular I Onset (hours)
Acetylcholine[1]	seconds
Carbachol	1
Pilocarpine	
Solution	0.75 to 1
Gel	1
Ocular system	1

[1] Intraocular administration.

Miosis following topical application of pil minutes and lasts 4 to 8 hours.

Indications:
To decrease elevated IOP in glaucoma.
Acetylcholine is used only for intraocular
Unlabeled use: Pilocarpine, 5 mg orally, mouth) in patients with malfunctioning

Contraindications:
Hypersensitivity to any component of the constriction are undesirabl (eg, acute block glaucoma, acut inflammatory d

Warnings:
Corneal abrasion: Use carbachol with ca excessive penetration.
Pregnancy: Category C (pilocarpin). Sa lished. Use only when clearly needed potential hazards to the fetus.
Lactation: It is not known whether these when administering to a nursing wom
Children: Safety and efficacy for use in c

Precautions:
Systemic reactions rarely occur during t acute angle-closure glaucoma, consic relatively high dosage required over a Caution is advised, although systen with acute cardiac failure, bronchial a nary tract obstruction and Parkinson's
Retinal detachment has been caused by with preexisting retinal disease or in examination is advised for all patients
Miosis usually causes difficulty in dark night driving or performing hazardou
Pilocarpine: Use with caution in patient closure.
Pilocarpine ocular system (Ocusert) – C infectious conjunctivitis or keratitis p

(Continu

US005559151A

United States Patent [19]

Adorante et al.

[11] Patent Number: 5,559,151

[45] Date of Patent: Sep. 24, 1996

[54] **METHOD FOR REDUCING INTRAOCULAR PRESSURE IN THE MAMMALIAN EYE BY ADMINISTRATION OF CHLORIDE CHANNEL BLOCKERS**

[75] Inventors: **Joseph S. Adorante**, Irvine; **Elizabeth WoldeMussie**, Laguna Niguel; **Guadalupe Ruiz**, Corona, all of Calif.

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[21] Appl. No.: **346,660**

[22] Filed: **Nov. 30, 1994**

[51] **Int. Cl.**[6] **A61K 31/195**

[52] **U.S. Cl.** **514/567**; 514/569; 514/737; 514/516; 514/912; 514/913; 424/78.04

[58] **Field of Search** 514/912, 913, 514/567, 569, 737, 516, 912, 913; 424/78.04

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Biochimica et al Biophysica Acta, vol. 947, 1988, pp. 521–547, Gogelein, H., "Chloride channels in epithelia".

American Physiological Society, 1994, vol. 0363–6143, pp. C1210–C–1221, Edelman et al., "Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells".

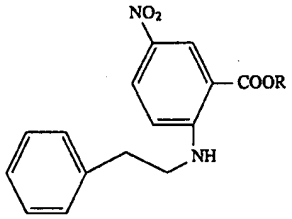
Acta Physiol Scand: No. 149, 1993: pp. 365–376, Fryklund et al., "The effects of chloride transport inhibitors on intestinal fluid and ion transport in vivo and in vitro".

Primary Examiner—James H. Reamer

Attorney, Agent, or Firm—Robert J. Baran; Martin A. Voet; Howard R. Lambert

[57] **ABSTRACT**

Pharmaceutical compositions and a method are disclosed for treating glaucoma and/or ocular hypertension in the mammalian eye by administering to the mammalian eye the pharmaceutical composition of the invention which contains, as the active ingredient, one or more compounds having chloride channel blocking activity. Examples of chloride channel blockers utilized in the pharmaceutical composition and method of treatment are:



wherein R is hydrogen or a pharmaceutically-acceptable cation, e.g. an alkali or alkaline earth metal, or a quaternary amine; or R represents a ester-forming moiety, e.g. a lower alkyl radical, having up to six carbon atoms, that may be derived from a lower alkanol.

10 Claims, 2 Drawing Sheets

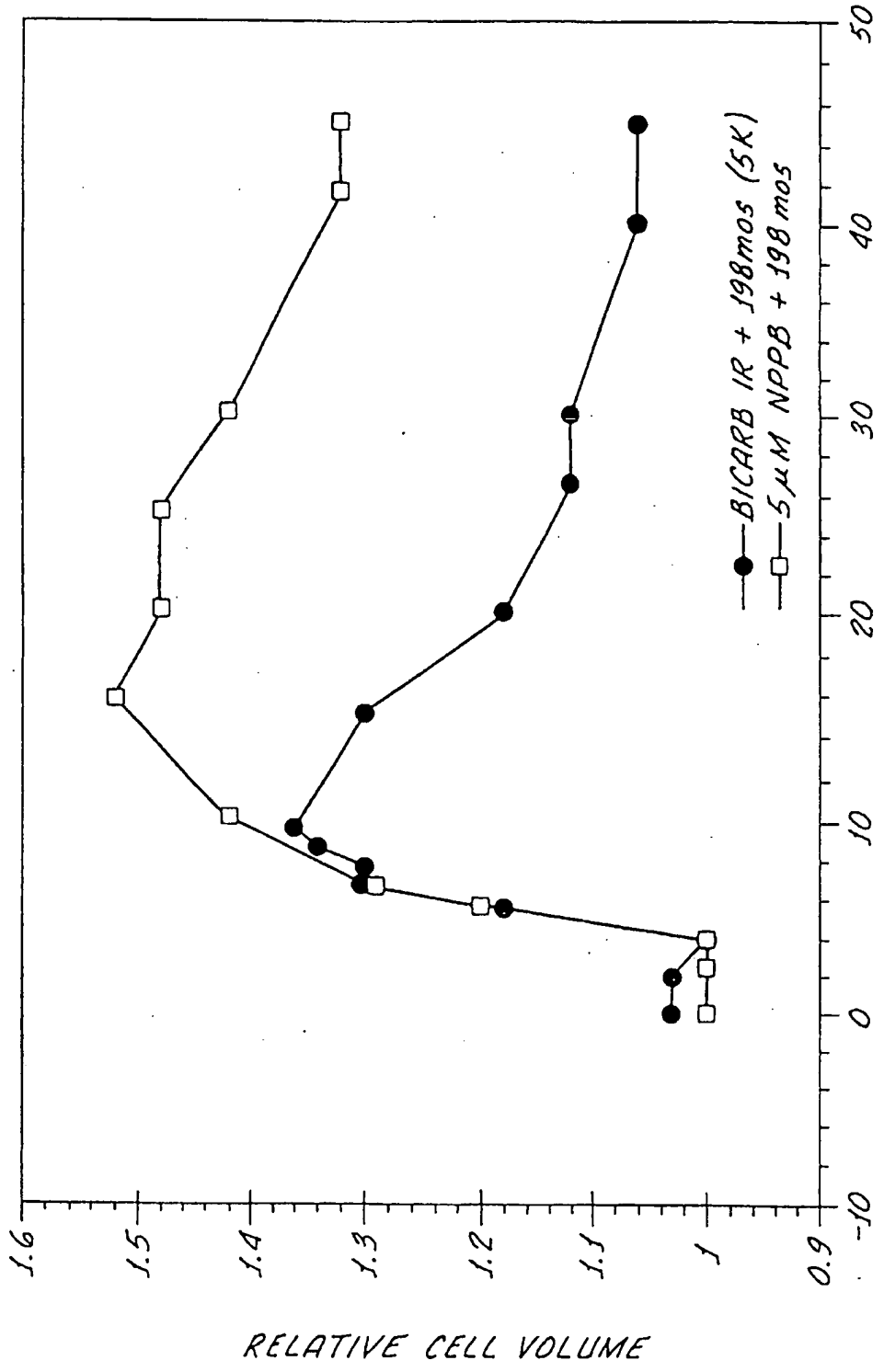

1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
-10
0
10
20
30
40
50
TIME (MIN.)
RELATIVE CELL VOLUME
BICARB IR + 198mos (5K)
5 μM NPPB + 198 mos

FIG. 1.

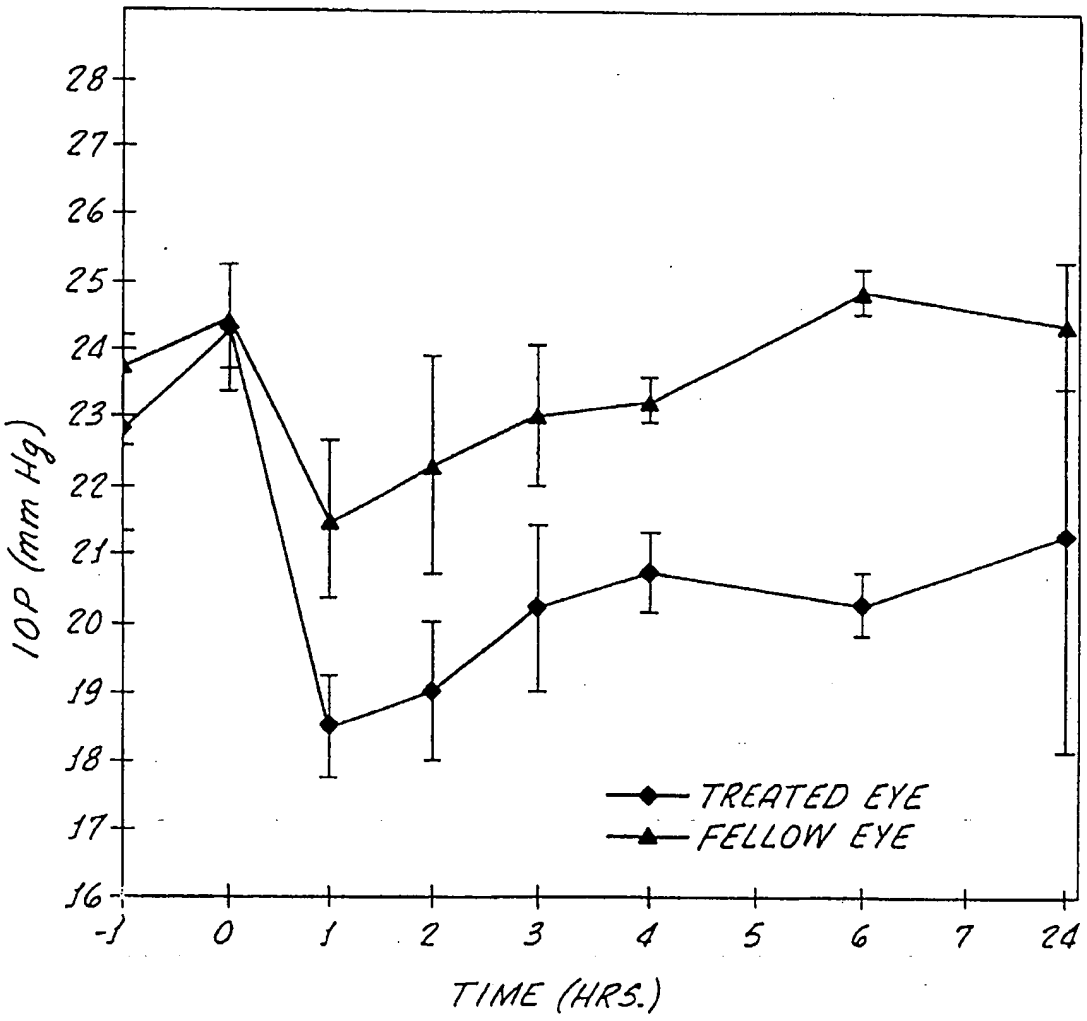

28
27
26
25
24
23
22
21
20
19
18
17
16
IOP (mm Hg)
-1
0
1
2
3
4
5
6
7
24
TIME (HRS.)
TREATED EYE
FELLOW EYE

FIG. 2.

METHOD FOR REDUCING INTRAOCULAR PRESSURE IN THE MAMMALIAN EYE BY ADMINISTRATION OF CHLORIDE CHANNEL BLOCKERS

BACKGROUND OF INVENTION

1. Field of the Invention

The present invention is directed to pharmaceutical compositions, and primarily to topically applied ophthalmic compositions comprising as the active ingredient one or more compounds having the ability to block chloride channels in the ciliary epithelium, e.g. to inhibit the transport of chloride ions and fluid secretion in epithelia. The pharmaceutical compositions are useful for reducing intraocular pressure in animals of the mammalian species. In another aspect, the present invention is directed to administering such formulations and compositions to animals of the mammalian species (including humans) for reducing intraocular pressure in the eye.

2. Brief Description of the Art

Glaucoma is an optical neuropathy associated with elevated intraocular pressures which are too high for normal function of the eye, and results in irreversible loss of visual function. It is estimated in medical science that glaucoma afflicts approximately 2 percent of the population over the age of forty years, and is therefore a serious health problem. Ocular hypertension, i.e. the condition of elevated intraocular pressure, which has not yet caused irreversible damage, is believed to represent the earliest phase of glaucoma. Many therapeutic agents have been devised and discovered in the prior art for the treatment or amelioration of glaucoma and of the condition of increased intraocular pressure which precedes glaucoma.

The drugs currently utilized in the treatment of glaucoma include miotics (e.g., pilocarpine, carbachol, and acetylcholinesterase inhibitors), sympathomimetrics (e.g., epinephrine and dipivalylepinephrine), beta-blockers (e.g., betaxolol, levobunolol and timolol), alpha-2 agonists (e.g., paraamino clonidine) and carbonic anhydrase inhibitors (e.g., acetazolamide, methazolamide and ethoxzolamide). Miotics and sympathomimetics are believed to lower intraocular pressure by increasing the outflow of aqueous humor, while beta-blockers, alpha-2 agonists and carbonic anhydrase inhibitors are believed to lower intraocular pressure by decreasing the formation of aqueous humor. All five types of drugs have potential side effects. Miotics, such as pilocarpine, can cause blurring of vision and other visual side effects which may either decrease patient compliance or require termination of miotic drug therapy. Carbonic anhydrase inhibitors can also cause serious side effects which affect patient compliance and/or necessitate withdrawal of the drug therapy. At least one beta-blocker, timolol, has increasingly become associated with serious pulmonary side effects attributable to its effect on beta-2 receptors in pulmonary tissue.

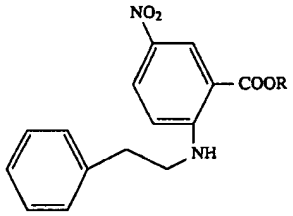
As a result additional antiglaucoma drugs are being developed, e.g., prostaglandin derivatives, muscarinic antagonists, etc.

In light of the foregoing circumstances, it is clear that a need exists for new, more potent antiglaucoma compositions which avoid or reduce the above-cited side effects and enhance patient compliance, since the foregoing and other anti-glaucoma and ocular hypotensive compounds and agents of the prior art do not provide a treatment or cure for glaucoma and ocular hypertension which is satisfactory in all respects. Therefore, the pharmacological and related arts and sciences continue searching for additional and better anti-glaucoma and ocular hypotensive agents.

Chloride channel blockers such as 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) have been shown to inhibit Cl^- transport and fluid secretion/absorption in rat intestine. (See for example, Acta Physiol Scand: No. 149, 1993: pp. 365–376, Fryklund et al., "The effects of chloride transport inhibitors on intestinal fluid and ion transport in vivo and in vitro".)

SUMMARY OF THE INVENTION

Surprisingly it has been discovered in accordance with the present invention that chloride channel blockers are effective as anti-glaucoma agents and as agents for reducing intraocular pressure, when such agents are applied to the mammalian eye in a pharmaceutical composition, preferably in a topical ophthalmic composition. Accordingly, the present invention relates to a method of treating glaucoma, or ocular hypertension by topically administering to the mammalian eye an ophthalmic composition which contains an effective amount of a chloride channel blocker. A preferred example of chloride channel blockers suitable as the active ingredients of the ophthalmic compositions of the invention are:



wherein R is hydrogen or a pharmaceutically-acceptable cation, e.g. an alkali ion, or a quarternary amine; or R represents a ester-forming moiety, e.g. a lower alkyl radical, having up to six carbon atoms, i.e. a radical that may be derived from a lower alkanol.

While not wishing to be bound by theory it is believed that in ciliary epithelium, i.e. the tissue mediating aqueous humor secretion, movement of fluid into the aqueous chamber, is in part orchestrated by K^+ and Cl^- channels residing in the nonpigmented (NPE) cells. (See for example, American Journal of Physiology, 1994, Vol. 0363–6143, pp C1210–C-1221, Edelman et al, "Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells".) Thus, aqueous secretion is inhibited and hence intraocular pressure (IOP) is lowered by blocking Cl^- channels in the NPE cells.

The ophthalmic compositions of the invention contain the active ingredient in a concentration range of approximately 0.0001 to 0.1 percent weight by volume. The composition itself includes, in addition to the active ingredient, such excipients which are per se well known in the art for preparing ophthalmic compositions, particularly ophthalmic solutions. In accordance with the method of the invention the ophthalmic compositions, preferably ophthalmic solutions are applied topically to the mammalian eye approximately 1 or 2 times daily.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the effect of the presence of the drug NPPB on the regulatory volume decrease (RVD) of a

suspension of cultured human non-pigmented ciliary epithelial (NPE) cells.

FIG. 2 is a graph showing the effect of intracameral administration of the drug NPPB on the intraocular pressure (IOP) in the rabbit eye.

DETAILED DESCRIPTION OF THE INVENTION

The compounds which are utilized in accordance with the method of the present invention, and in the pharmaceutical compositions of the present invention, are chloride channel blockers. In this regard the term chloride channel blocker is defined as those compounds or agents which inhibit net Cl flux (current) through a Cl specific pathway (channel, integral membrane protein) within biological membranes. Specific and preferred examples of chloride channel blockers which are utilized in accordance with the present invention are provided below.

Pharmaceutically acceptable salts of the chloride channel blockers can also be used in accordance with the present invention. A pharmaceutically acceptable salts may be any salt which retains the activity of the parent compound and does not impart any deleterious or untoward effect on the subject to which it is administered and in the context in which it is administered.

Such a salt may be derived from any organic or inorganic acid or base. The salt may be a mono or polyvalent ion. Of particular interest where the acid function is concerned are the inorganic ions, such as alkali ions, e.g. sodium, potassium, etc. Organic amine salts may be made with amines, particularly ammonium salts such as mono-, di- and trialkyl amines, e.g. alkyl amines wherein each alkyl group may comprise up to six carbon atoms, or ethanol amines. Salts may also be formed with caffeine, tromethamine and similar molecules. It is only important that the cation of any salt of a chloride channel blocker utilized in the compositions or methods of this invention be able to block chloride channels in the ciliary epithelium.

For reducing intraocular pressure in a mammalian eye, and particularly for treatment of glaucoma in humans suffering from that condition, the active compounds (or mixtures or salts thereof) are administered in accordance with the present invention to the eye admixed with an ophthalmically acceptable carrier. Any suitable, e.g., conventional, ophthalmically acceptable carrier may be employed. A carrier is ophthalmically acceptable if it has substantially no long term or permanent detrimental effect on the eye to which it is administered. Examples of ophthalmically acceptable carriers include water (distilled or deionized water), saline and other aqueous media. In accordance with the invention, the active compounds are preferably soluble in the carrier which is employed for their administration, so that the active compounds are administered to the eye in the form of a solution. Alternatively, a suspension of the active compound or compounds (or salts thereof) in a suitable carrier may also be employed.

In accordance with the invention the active compounds (or mixtures or salts thereof) are administered in an ophthalmically acceptable carrier in sufficient concentration so as to deliver an effective amount of the active compound or compounds to the eye. Preferably, the ophthalmic, therapeutic solutions contain one or more of the active compounds in a concentration range of approximately 0.0001% to approximately 1% (weight by volume) and more preferably approximately 0.0005% to approximately 0.1% (weight by volume).

Any method of administering drugs directly to a mammalian eye may be employed to administer, in accordance with the present invention, the active compound or compounds to the eye to be treated. By the term "administering directly" is meant to exclude those general systemic drug administration modes, e.g., injection directly into the patient's blood vessels, oral administration and the like, which result in the compound or compounds being systemically available. The primary effect on the mammal resulting from the direct administering of the active compound or compounds to the mammal's eye is preferably a reduction in intraocular pressure. More preferably, the active useful compound or compounds are applied topically to the eye or are injected directly in to the eye. Particularly useful results are obtained when the compound or compounds are applied topically to the eye in an ophthalmic solution, i.e. as ocular drops.

Topical ophthalmic preparations, for example ocular drops, gels or creams, are preferred because of ease of application, ease of dose delivery and fewer systemic side effects, such as cardiovascular hypotention. An exemplary topical ophthalmic formulation is shown below in Table I. The abbreviation q.s. means a quantity sufficient to effect the result or to make volume.

TABLE I

Ingredient	Amount (% W/V)
Active Compound in accordance with the invention,	about 0.0001 to about 1
Preservative	0–0.10
Vehicle	0–40
Tonicity Adjustor	1–10
Buffer	0.01–10
pH Adjustor	q.s. pH 4.5–7.5
antioxidant	as needed
Purified Water	as needed to make 100%

Various preservatives may be used in the ophthalmic preparation described in Table I above. Preferred preservatives include, but are not limited to, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric acetate, and phenylmercuric nitrate. Likewise, various preferred vehicles may be used in such ophthalmic preparation. These vehicles include, but are not limited to, polyvinyl alcohol, povidone, hydroxypropyl methyl cellulose, poloxamers, carboxymethyl cellulose and hydroxyethyl cellulose.

Tonicity adjustors may be added as needed or convenient. They include, but are not limited to, salts, particularly sodium chloride, potassium chloride etc., mannitol and glycerin, or any other suitable ophthalmically acceptable tonicity adjustor.

Various buffers and means for adjusting pH may be used so long as the resulting preparation is ophthalmically acceptable. Accordingly, buffers include but are not limited to, acetate buffers, citrate buffers, phosphate buffers, and borate buffers. Acids or bases may be used to adjust the pH of these formulations as needed.

In a similar vein, ophthalmically acceptable antioxidants include, but are not limited to, sodium metabisulfite, sodium thiosulfate, acetylcysteine, butylated hydroxyanisole, and butylated hydroxytoluene.

The ophthalmic solution (ocular drops) may be administered to the mammalian eye as often as necessary to maintain an acceptable level of intraocular pressure in the eye. In other words, the ophthalmic solution (or other formulation) which contains the chloride channel blocker as the active

ingredient, is administered to the mammalian eye as often as necessary to maintain the beneficial hypotensive effect of the active ingredient in the eye. Those skilled in the art will recognize that the frequency of administration depends on the precise nature of the active ingredient and its concentration in the ophthalmic formulation. Within these guidelines it is contemplated that the ophthalmic formulation of the present invention will be administered to the mammalian eye approximately once or twice daily.

Specific examples of chloride channel blockers which are used as the active effective ingredients in the ophthalmic compositions of the present invention are described and shown below: N-phenylanthranilic acid, DPC (diphenylamine-2-carboxylic acid), IAA-94 (R(+)-methylindazone, indanyloxyacetic acid 94), 2-aminomethyl phenols such as MK-447 (2-aminomethyl-4-(1,1-dimethyl ethyl)-6-iodophenol hydrochloride (2) disulfonic stilbenes such as DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid).

Alternatively, a chloride channel blocker may be defined as a pharmaceutical compound showing activity in the following assay:

Inhibition of current mediated by Cl ions using patch clamp technology in the whole cell, cell attached or cell excised mode. (See for Example, Biochimica et al Biophysica Acta, Vol. 947, 1988, pp. 521–547, Gogelein, H., "Chloride channels in epithelia".)

EXAMPLES

The present invention is demonstrated by in vitro and in vivo data. In FIG. **1**, 5 μM NPPB were found to depress the regulatory volume decrease (RVD) that occurs following hyposmotic swelling of cultured human non-pigmented ciliary epithelial (NPE) cells. In this example, NPE cells were suspended in an isosmotic (290 mOsm) solution containing 5 μM NPPB for 30 minutes prior to suspension in a hyposmotoic (198 mOsm) solution. Control cells were subjected to the same hyposmotic solution but without NPBB in the medium. Changes in cell volume were measured using a Coulter Counter interfaced to a Coulter Channelyzer. It is noted that, following osmotic swelling, control cells regulate towards their original isosmotic volume while NPBB-treated cells remain swollen. The above findings indicate that intracellular NPBB, via blocking of the chloride channel, inhibits solute and osmotically obliged H_2O efflux. Because the chloride-dependent ion flux pathways, activated following osmotic cell swelling of NPE cells, are involved in aqueous secretion, NPBB will inhibit aqueous humor formation and, thus, lower IOP.

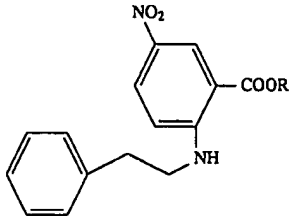
In the in vivo studies normotensive rabbits were injected intracamerally with 100 μM NPBB. FIG. **2** shows that 100 μM NPBB lowered IOP by 7 mm of Hg and IOP remained depressed for 24 hours. Taken together, the above in vitro and in vivo experiments demonstrate that blocking the chloride ion channel in the ciliary epithelium will reduce IOP.

One advantage Cl^- channel inhibition has over other IOP lowering therapies is that the effector, i.e. the ion channel, is targeted rather than the receptor. Since effector blockage is direct, it should be the most potent and effective way of inhibiting aqueous secretion and hence lowering IOP. On the other hand, targeting a receptor to block an effector is indirect and relies on modulation of a series of cellular events (intracellular messengers/signals) prior to effector inhibition.

In view of the above, it is clear that the scope of the present invention should be interpreted solely on the basis of the following claims, as such claims are read in light of the disclosure.

What is claimed is:

1. A method of treating animals of the mammalian species, including humans, for the purpose of reducing introacular pressure in the eye of the mammal comprising the step of administering to the mammal a pharmaceutical composition which comprises as its active ingredient a compound having chloride channel blocking activity, wherein said compound is selected from the group consisting of compounds represented by the formula:



wherein R is hydrogen or a pharmaceutically-acceptable cation of R represents a easter-forming moiety.

2. The method of treatment of claim **1** where the composition is an ophthalmic solution adapted for administration to the eye of a mammal in the form of eye droplets.

3. The method of treatment of claim **2** wherein in the ophthalmic composition the concentration of the compound having chloride channel blocking activity is in the range of approximately 0.0001 to 1 percent weight by volume.

4. A method of treating animals of the mammalian species, including humans, for the purpose of reducing intraocular pressure in the eye of the mammal comprising the step of administering to the mammal an ophthalmic composition which comprises as its active ingredient one or more compounds having chloride channel blocking activity compounds selected from the group consisting of N-phenylanthranilic acid, DPC (diphenylamine-2-carboxylic acid), IAA-94 (R(+)-methylindazone, indanyloxyacetic acid 94), 2-aminomethyl phenols such as MK-447 (2-aminomethyl-4-(1,1-dimethyl ethyl)-6-iodophenol hydrochloride (2) disulfonic stilbenes such as DIDS (4,4'-diisothiocyanostilbene-2-2'-disulfonic acid) and 5-nitro-2(3 phenyl propyl amino)-benzoate (NPPB).

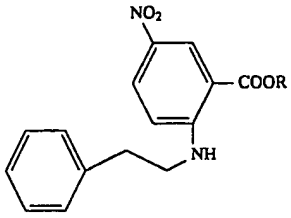
5. The method of treatment of claim **4** wherein the composition is an ophthalmic solution adapted for administration to the eye of a mammal in the form of eye droplets.

6. The method of claim **1** wherein R is hydrogen, or an alkali metal ion, or a quaternary amine selected from the group consisting of mono-, di- and trialkyl amines, wherein each alkyl group may comprise up to six carbon atoms, or ethanol amine, or caffeine, or tromethamine, or a lower alkyl radical having up to six carbon atoms.

7. The method of treatment of claim **5** wherein in the ophthalmic composition the concentration of the compound having chloride channel blocking activity is in the range of approximately 0.0001 to 1 percent weight by volume.

8. A method for providing neuroprotective effect to the eye of a mammal which comprises the step of administering to the mammal a pharmaceutical composition which comprises as its active ingredient one or more compounds having chloride channel blocking activity wherein said compound having a chloride channel blocking activity is selected from the group consisting of N-phenylanathracilic acid, DPC (diphenylamine-2-carboxylic acid), IAA-94 (R+)-methylidazone, indanyloxyacetic, acid 94), MK-447 (2-aminoemethyl-4-(1,1 -dimethyl ethyl)-6-iodophenol

hydrochloride, DIDS (4,4'-diisothiocyanostilbene- 2,2'-disulfonic acid) and compounds represented by the formula:



wherein R is hydrogen or a pharmaceutically-acceptable cation, or R represents a ester-forming moiety.

9. The method of claim **8** wherein the composition contains approximately 0.0001 to 1 percent weight by volume of said compound having chloride channel blocking activity.

10. The method of claim **8** wherein R is hydrogen, or an alkali metal ion, or a quaternary amine selected from the group consisting of mono-, di- and trialkyl amines, wherein each alkyl group may comprises up to six carbon atoms, or ethanol amine, or caffeine, or tromethamine, or a lower alkyl radical having up to six carbon atoms.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,559,151

DATED : 9/24/1996

INVENTOR(S) : Adorante et al

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 2, line 46; delete "nonpigmented" and insert in place thereof --non-pigmented ciliary epithelial--

Column 4, line 14; delete "in to" and insert in place thereof --into--

Column 5, lines 29 and 30; put in italics "in vivo" and "in vitro"

Column 5, lines 50, 53 and 54; put in italics "in vivo" and "in vitro"

Column 3, line 21; delete "salts" and insert in place thereof --salt--

Column 5, line 24; delete "Example" and insert in place thereof --example--

Column 6, line 22; delete "of" and insert in place thereof --or--

Column 6, line 22; delete "easter" and insert in place thereof --ester--

Column 6, line 41; delete "(2)" and insert in place thereof --or--

Column 6, line 64; delete "phenylanathracilic" and insert in place thereof --phenylanthranilic--

Column 6, line 66; delete "methylidazone" and insert in place thereof --methylindazone--

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,559,151 Page 2 of 2

DATED : 9/24/1996

INVENTOR(S) : Adorante et al

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 6, line 67; delete "aminoemethyl" and insert in place thereof --aminomethyl--

Column 8, line 10; delete "comprises" and insert in place thereof --comprise--

Signed and Sealed this

Eighteenth Day of March, 1997

Bruce Lehman

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

US005585401A

United States Patent [19]

Brandt et al.

[11] **Patent Number: 5,585,401**

[45] **Date of Patent: Dec. 17, 1996**

[54] **METHOD FOR ENHANCING OUTFLOW OF AQUEOUS HUMOR IN TREATMENT OF GLAUCOMA**

[75] Inventors: **James D. Brandt**, Folsom; **Martha E. O'Donnell**; **Fitz-Roy E. Curry**, both of Davis, all of Calif.

[73] Assignee: **The Reents of the University of California**, Oakland, Calif.

[21] Appl. No.: **353,442**

[22] Filed: **Dec. 9, 1994**

[51] **Int. Cl.**^{6} **A61K 31/19**

[52] **U.S. Cl.** **514/562**

[58] **Field of Search** 514/562

[56] **References Cited**

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Primary Examiner—Phyllis G. Spivack

Attorney, Agent, or Firm—Fish & Richardson P.C.

[57] **ABSTRACT**

Elevated intraocular pressure is reduced by administration directly to the eye of compounds that inhibit function of a Na^{+}—K^{+}—$2Cl^{-2}$ cotransporter mechanism discovered in trabecular meshwork cells of mammalian eyes. These compounds are useful in treatment of diseases of the eye associated with elevated intraocular pressure, such as ocular hypertension and glaucoma. A screening method is provided to discover additional compounds with utility for lowering intraocular pressure by substantially inhibiting the Na^{+}—K^{+}—Cl^{-2} cotransporter mechanism in trabecular meshwork cells.

18 Claims, 6 Drawing Sheets

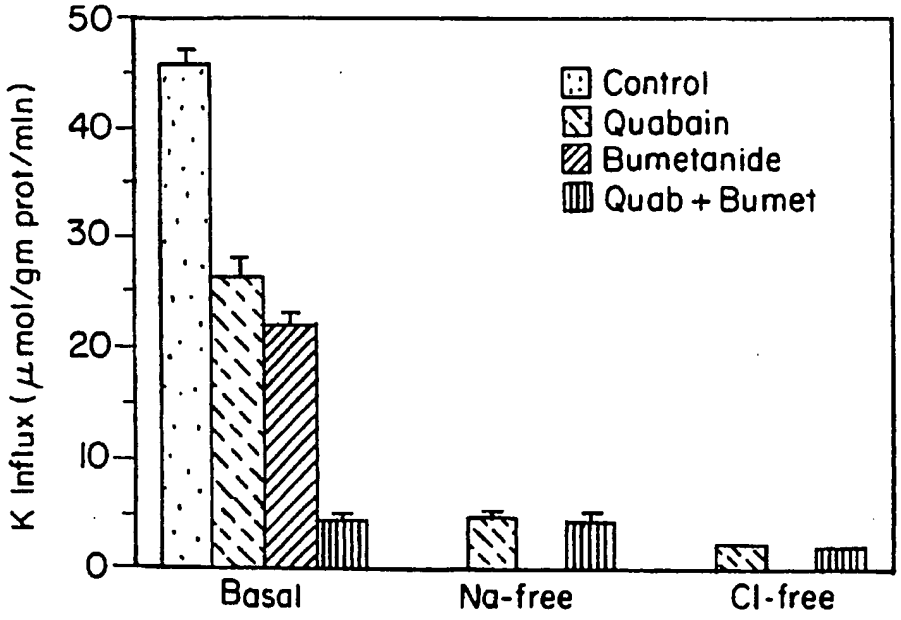

50
40
30
20
10
0
K Influx (μmol/gm prot/min)
Control
Quabain
Bumetanide
Quab + Bumet
Basal
Na-free
Cl-free

FIG. 1A

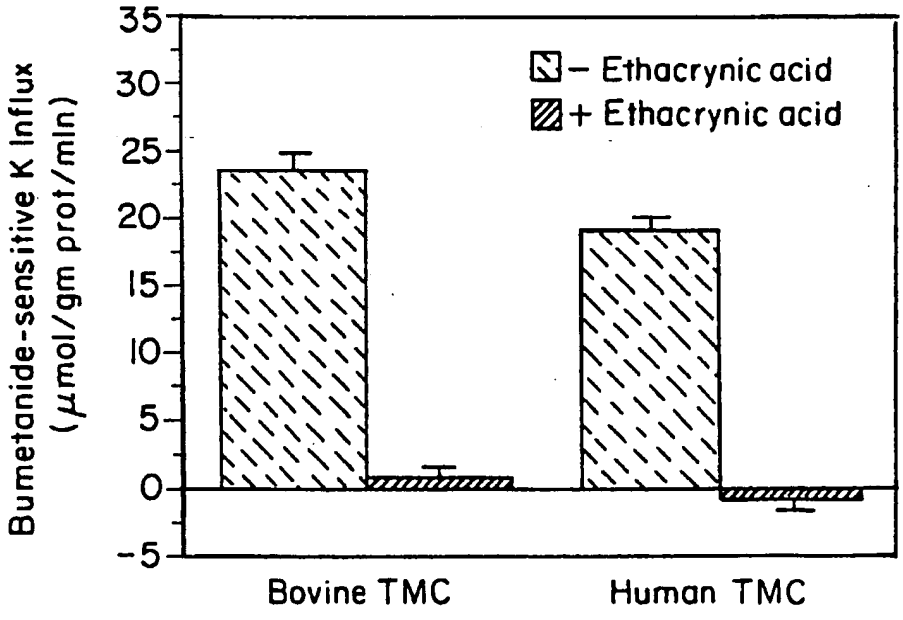

35
30
25
20
15
10
5
0
-5
Bumetanide-sensitive K Influx (μmol/gm prot/min)
– Ethacrynic acid
+ Ethacrynic acid
Bovine TMC
Human TMC

FIG. 1B

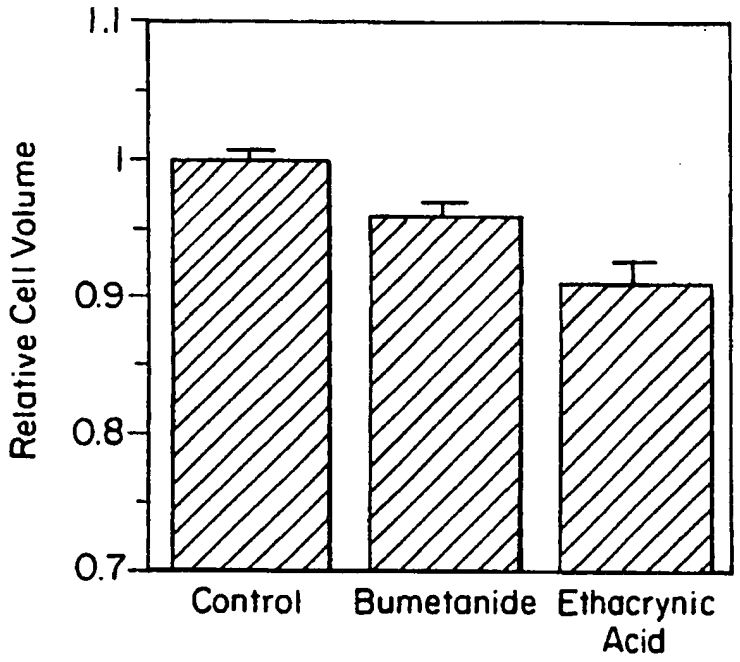

1.1
1
0.9
0.8
0.7
Relative Cell Volume
Control
Bumetanide
Ethacrynic Acid

FIG. 2A

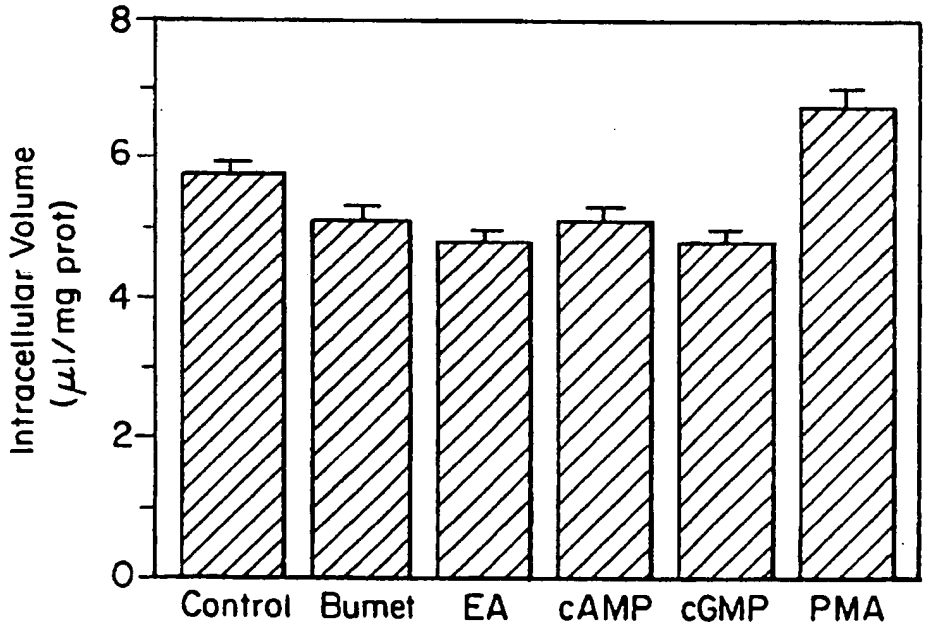

8
6
4
2
0
Intracellular Volume (μl/mg prot)
Control
Bumet
EA
cAMP
cGMP
PMA

FIG. 2B

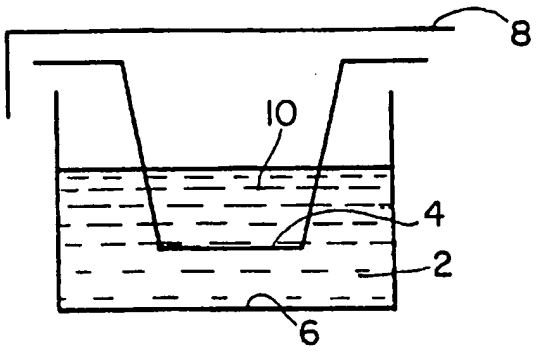

8
10
4
2
6

FIG. 3A

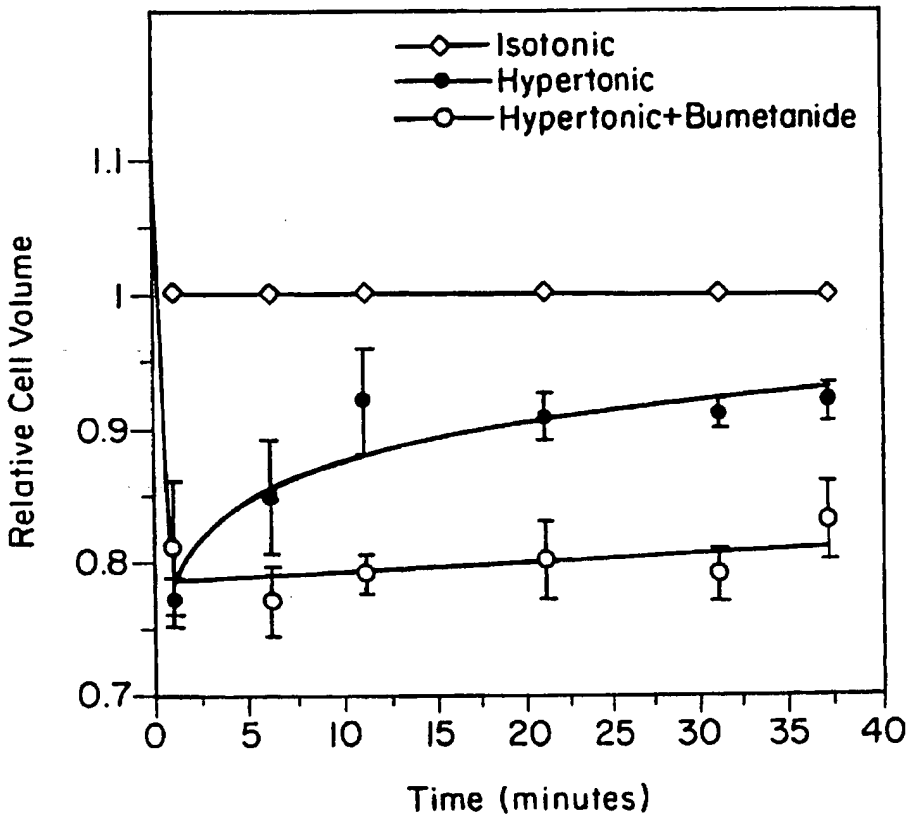

Isotonic
Hypertonic
Hypertonic+Bumetanide
Relative Cell Volume
1.1
1
0.9
0.8
0.7
0
5
10
15
20
25
30
35
40
Time (minutes)

FIG. 3B

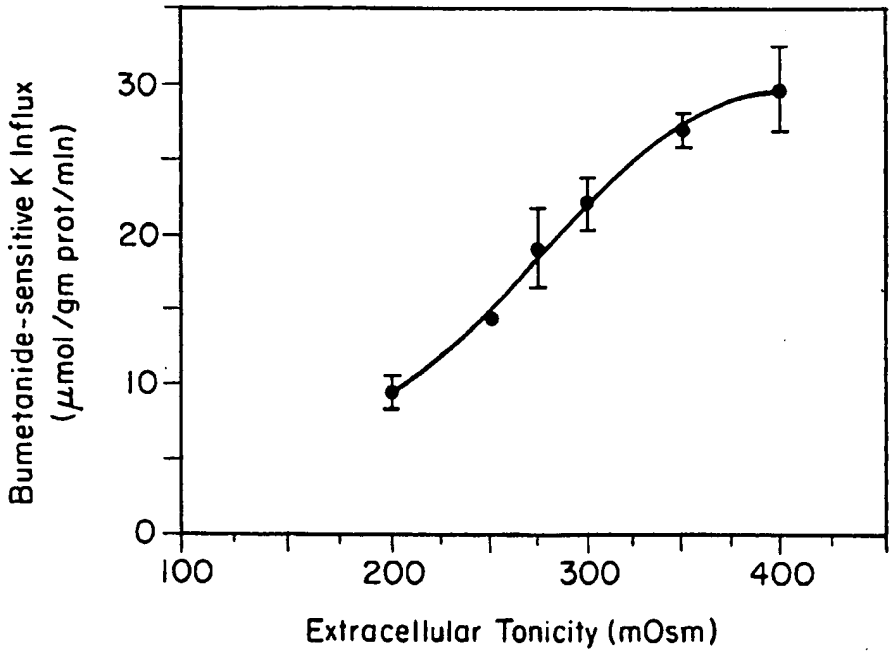

Bumetanide-sensitive K Influx
(μmol/gm prot/min)
30
20
10
0
100
200
300
400
Extracellular Tonicity (mOsm)

FIG. 3C

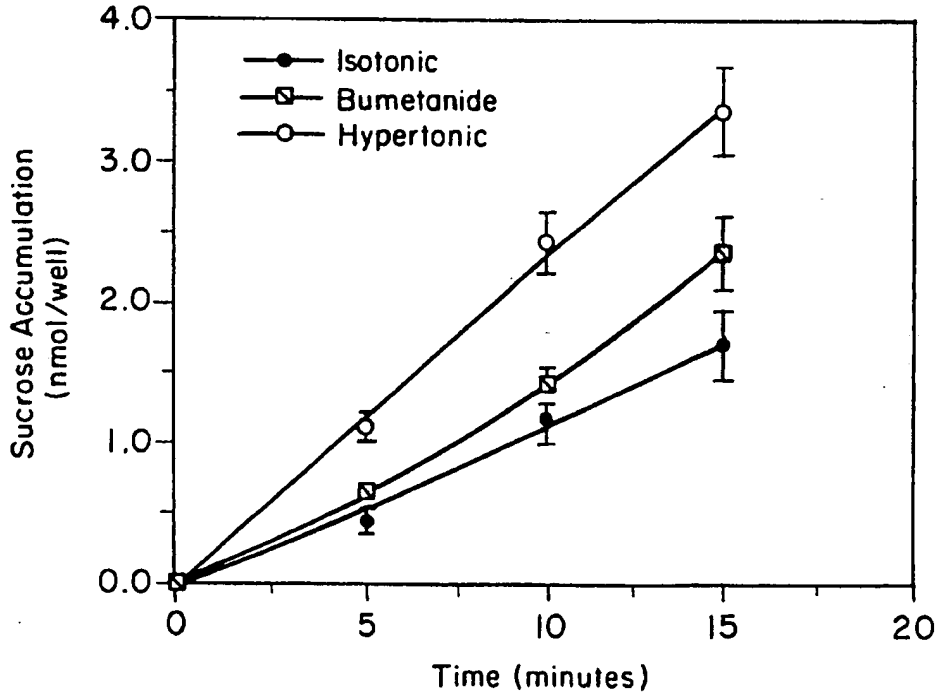

Sucrose Accumulation
(nmol/well)
4.0
3.0
2.0
1.0
0.0
Isotonic
Bumetanide
Hypertonic
0
5
10
15
20
Time (minutes)

FIG. 4

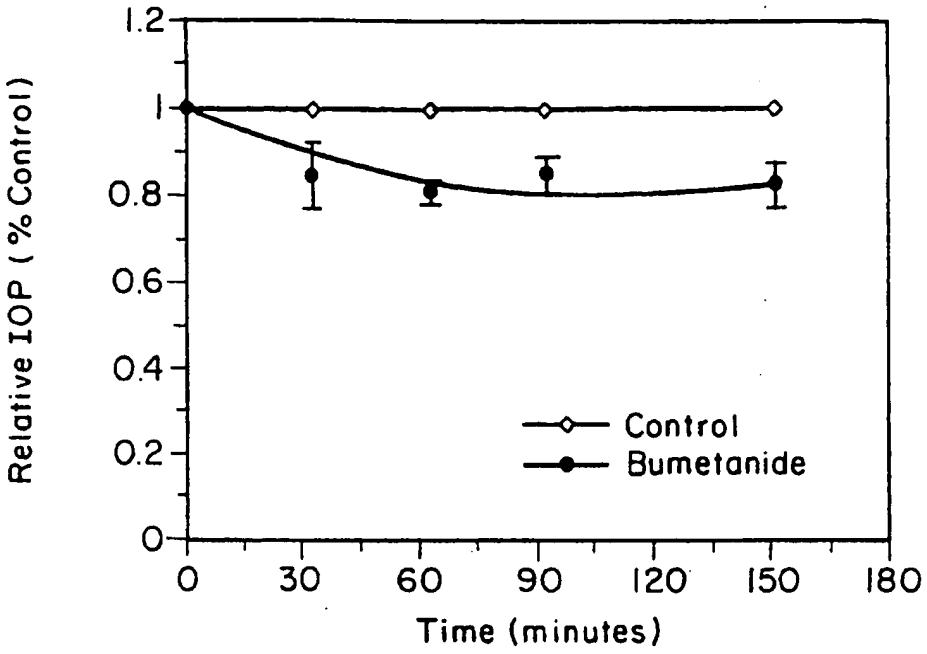

1.2
1
0.8
0.6
0.4
0.2
0
Relative IOP (% Control)
Control
Bumetanide
0
30
60
90
120
150
180
Time (minutes)

FIG. 5A

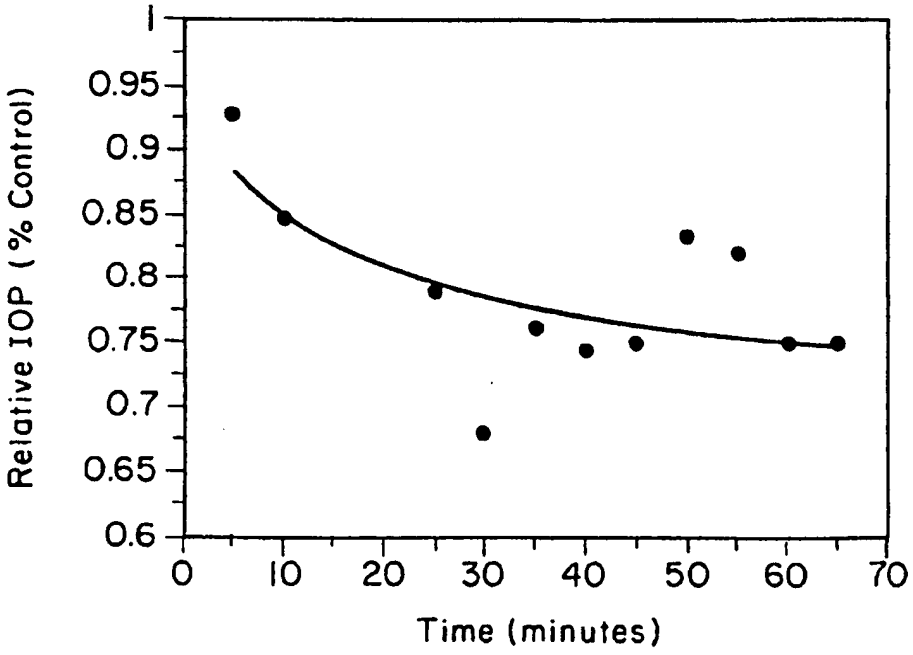

1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
Relative IOP (% Control)
0
10
20
30
40
50
60
70
Time (minutes)

FIG. 5B

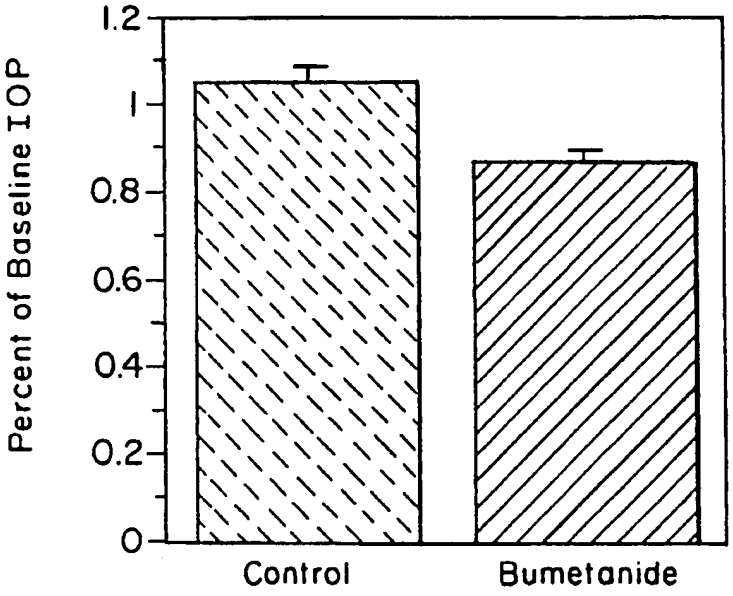

Percent of Baseline IOP
1.2
1
0.8
0.6
0.4
0.2
0
Control
Bumetanide

FIG. 6

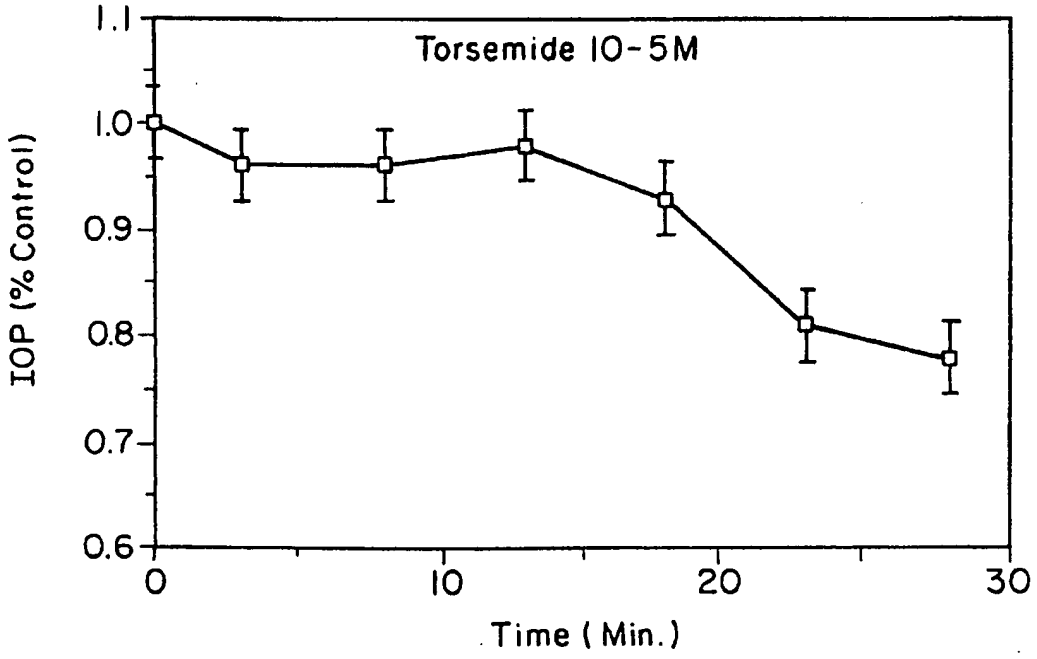

Torsemide 10-5M
IOP (% Control)
1.1
1.0
0.9
0.8
0.7
0.6
0
10
20
30
Time (Min.)

FIG. 7

METHOD FOR ENHANCING OUTFLOW OF AQUEOUS HUMOR IN TREATMENT OF GLAUCOMA

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to methods for reducing the intraocular pressure of the eye by enhancing aqueous humor outflow, and to a method for screening compounds that reduce intraocular pressure.

2. Description of Related Art

In glaucoma, a leading cause of blindness, the optic nerve is damaged through a poorly-understood interaction of elevated intraocular pressure (IOP) and patient predisposition to the disease. In the most common form of glaucoma the trabecular meshwork (TM), which plays a critical role in regulation of aqueous humor outflow and intraocular pressure in both health and disease, is thought to be defective in such a manner that resistance to outflow and IOP both rise.

The anterior chamber of the eye is bathed with aqueous humor, formed continuously by the ciliary body. Aqueous humor moves by bulk flow from its site of production in the posterior chamber through the pupillary aperture and into the anterior chamber. It subsequently exits the anterior chamber via one of two routes. The majority of outflow in the healthy human eye occurs at the anterior chamber angle, where aqueous humor passes through the trabecular meshwork and into the Canal of Schlemm, from where it joins the general venous drainage of the eye. A second outflow pathway is via the uveoscleral route, although this appears to be a minor (≈20%) pathway in the normal human eye. A homeostatic balance of aqueous humor production and drainage allows intraocular pressure to be maintained within narrow limits in the normal eye (Caprioli, J., *Adler's Physiology of the Eye: Clinical Application*, W. M. Hart, ed. 9th Ed., 7:228–247, 1992; Hart, W. M., *Adler's Physiology of the Eye: Clinical Application*, W. M. Hart, ed. 9th Ed., 8:248–267, 1992).

Production of aqueous humor occurs along the surface of the ciliary processes (pars plicata), which is covered by a double layer of epithelial cells consisting of a pigmented and non-pigmented layer situated with their apical surfaces juxtaposed. These function in tandem to produce transepithelial secretion of NaCl and water in movement from the blood to the aqueous humor. Evidence has been provided that Na—K—Cl cotransport and the Na/K pump act in concert to bring about the vectorial transport (Dong, et al., *Invest. Ophthalmol. Vis. Sci.*, 35:1660, 1994). The rate of aqueous humor production is quite high relative to other types of epithelia that function in vectorial transport of water and electrolytes. Thus, a drainage pathway that can accommodate this rate of fluid production is essential for maintenance of normal intraocular pressure. The aqueous humor production and drainage mechanisms work to replace the entire volume of aqueous every 100 minutes (Caprioli, J., supra).

It is well recognized that regulation of aqueous humor outflow through the trabecular meshwork is critically important for maintenance of an appropriate intraocular pressure, and that in disease states such as ocular hypertension and glaucoma, this regulation appears to be defective. For instance, U.S. Pat. No. 4,757,089 teaches a method for increasing aqueous humor outflow by topical or intracameral adminisation of ethacrynic acid, or an analog, to treat

glaucoma. It is also known that ethacrynic acid increases water flux across the walls of perfused microvessels (Brandt, et al., *Invest. Ophthalmol. Vis. Sci.*, 35(4[Suppl]):1848, 1994) and inhibits Na^+—K^+—$2Cl^-$ cotransport activity of avian erythrocytes (Palfrey, et al., *Am. J. Physiol.*, 264:C1270–C1277, 1993), although the mechnaisms by which these phenomena occur have not been elucidated. For instance, phenoxyacetic acids inhibit NaCl reabsorption in the thick ascending limb of the loop of Henle screening test, but its effect was exerted from both epithelial sides, rather than from the luminal side as with the class of loop diuretics, and it led to a depolarization of the membrane voltage. This effect is compatible with an inhibitory action at the level of mitochondrial ATP production rather than an inhibition of the Na^+—K^+—$2Cl^-$ cotransporter.

In contrast to the current level of knowledge regarding cellular processes responsible for aqueous humor production by the ciliary body, relatively little is known about the cellular mechanisms in the trabecular meshwork that determine the rate of aqueous outflow. Pinocytotic vesicles are observed in the juxtacanalicular meshwork and the inner wall of Schlemm's Canal. The function of these vesicles remains unknown, but some investigators have suggested that the bulk flow of aqueous humor through the meshwork cannot be accounted for by flow through the intercellular spaces and that these vesicles play a central role in outflow regulation. Evidence has been provided that cytoskeleton-mediated changes in trabecular meshwork cell shape modulate aqueous outflow (Erickson-Lamy and Nathanson, *Invest. Ophthalmol. Vis. Sci.*, 33:2672–2678, 1992; Erickson-Lamy, Schroder, and Epstein, *Invest. Opthalmol. Vis. Sci.*, 33:2631–2640, 1992). The extracellular matrix surrounding the trabeculae is thought to contribute to outflow resistance, perhaps by interactions with proteins contained in the aqueous humor (Freddo, T. F., *Optometry Vis. Sci.*, 70:263–270, 1993). Indeed, abnormalities in this extracellular matrix may contribute to the increased outflow resistance seen in corticosteroid-induced glaucoma (Partridge, et al., *Invest. Ophthalmol. Vis. Sci.*, 30:1843–1847, 1989; Polansky, et al., *The Ocular Effects of Prostaglandins and Other Eicosanoids*, Alan R. Liss, Inc., pp. 113–138, 1989). Investigators evaluating both normal physiology and drug effects have provided evidence that changes in cell shape (as distinct from cell volume) may be involved in outflow regulation (Erickson-Lamy and Nathanson, supra; Erickson-Lamy, Schroder, and Epstein, supra). Trabecular meshwork cells have been shown to possess actin and myosin filaments (Clark, et al., *Invest. Ophthalmol. Vis. Sci.*, 35:281–294, 1994) and to contract in response to some agents (Coroneo, et al., *Exp. Eye Res.*, 52:375–388, 1990; Lepple-Wienhues, et al., *Exp. Eye Res.*, 53:33–38, 1991; Wiederholt, et al., *Invest. Ophthalmol. Vis. Sci.*, 35:2515–2520, 1994). In a review of the existing literature at the time, Davson speculated that changes in trabecular meshwork cell volume (as distinct from cell shape) may participate in the regulation of aqueous outflow facility (Davson, H., *Physiology of the Eye*, H. Davson, ed., 5th Ed., Macmillan Press, London, Chapter 1, pp. 9–81, 1990), but to date this hypothesis has not been specifically addressed by other investigators. An excellent review of trabecular meshwork physiology and morphology is found in P. L. Kaufman, "Pressure-dependent Outflow" in R. Ritch et al., ed. *The Glaucomas*. St. Louis, Mo.:C. V. Mosby Co., 1989, 219–240, Vol. 1.

In addition to regulation of aqueous outflow, trabecular meshwork cells are thought to serve an immunologic function as they phagocytize antigens in the anterior chamber of the eye as they pass through the trabecular meshwork

(Epstein, et al., *Invest. Opthalmol. Vis. Sci.*, 27:387–395, 1986). It has been hypothesized that the cells then migrate out of the meshwork into the Canal of Schlemm to enter the systemic circulation and act as antigen presenting cells to trigger the production of antibodies to the phagocytized antigen. In at least one form of glaucoma (pigmentary), this phagocytotic function is thought to be overwhelmed, resulting in increased resistance to aqueous outflow (Epstein, et al., supra). The endothelial cells lining the Canal of Schlemm also appear to contribute to the resistance to outflow in the normal eye (Davson, H., supra; Hart, W. M., supra).

A number of hormones and neurotransmitters have been documented to decrease intraocular pressure by modulating aqueous production or outflow. Studies employing a human eye perfusion model have shown that epinephrine, via an apparent β-adrenergic effect upon the uveo-scleral pathway, increases the facility of outflow (Erickson-Lamy and Nathanson, supra). Nitrovasodilators have been found to increase outflow facility and decrease intraocular pressure in monkey eye (Schuman, et al., *Exp. Eye Res.*, 58:99–105, 1994). Similarly, atrial natriuretic peptide decreases intraocular pressure in monkey eyes and increases aqueous humor production (Samuelsson-Almen, et al., *Exp. Eye Res.*, 53:253–260, 1991). In addition to these hormones and neurotransmitters, ethacrynic acid has been shown to increase aqueous outflow and decrease intraocular pressure by modulating aqueous inflow and outflow. Elevations of norepinephrine concentration in the aqueous humor resulting from cervical sympathetic nerve stimulation cause an increase in intraocular pressure of rabbit eye in situ by a mechanism that appears to involve an α-adrenergic effect (Gallar, et al., *Invest. Ophthalmol. Vis. Sci.*, 34:596–605, 1993). Similarly, topical administration of vasopressia to the eye has been shown to increase intraocular pressure and decrease facility of outflow in both normal and glaucomatous human eyes (Becker, et al., *Arch. Ophthalmol.*, 56:1, 1956; Viggiano, et al., *Am. J. Ophthalmol.*, 115:511–516, 1993). A local renin-angiotensin system resides in the eye, and inhibition of angiotensin converting enzyme causes a decrease of intraocular pressure (Abrahms, et al., *J. Ocular Pharmacol.*, 7:41–51, 1991; Deinum, et al., *Endocrinol.*, 126:1673–1682, 1990). In contrast to these rapidly-acting agents, administration of the glucocorticoid dexamethasone increases resistance to outflow over a slower time course of hours and days, an effect that has been postulated to occur in the expression of extracellular matrix (Becker, et al., *Arch. Ophthalmol.*, 70:500–507, 1963; Clark, et al., supra; Partridge, et al., supra; Polansky, et al., supra).

Relatively little is known about the signal transduction and ion transport properties of TM cells. Cultured bovine trabecular meshwork cells have been examined for their ability to regulate intracellular pH (Chu, et al., *Acta Ophthalmol.*, 70:772–779, 1992). These studies demonstrated that the cells possess a Na/H exchanger that is activated by intracellular acidification and inhibited by amiloride, as is Na/H exchange of other cell types. In other studies of cultured bovine TM cells, Coroneo, et al.,supra, have provided electrophysiological evidence that these cells also possess Na/K ATPase and K channels. The presence of Ca channels in these TM cells has been indicated by the observation that the Ca channel blocker nifedipine prevents endothelin-evoked depolarization of the cells (Lepple-Wienhues, et al., *German J. Ophthalmol.*, 1:159–163, 1992). In addition, both plasma membrane and sarcolemmal Ca ATPases have been identified in rabbit TM cells by cytochemical methods (Kobayashi, et al., *Acta Soc. Ophthalmol. Jap.*, 93:396–403, 1989).

Na—K—Cl cotransport is a plasma membrane ion transport system found in a wide variety of cell types, both epithelial and non-epithelial (Chipperfield, A., *Clin. Sci.*, 71:465–476, 1986; Haas, M., *Ann. Rev. Physiol.*, 51:443–457, 1989; Pewitt, et al., *J. Biol. Chem.*, 265(34):20747–20756, 1990). It is a bidirectional transport mechanism, indicating that each transport molecule binds to the three transported ion species (Na, K and Cl), and moves them together across the plasma membrane in the same direction. The transporter is bidirectional such that it can operate to move the ions into or out of the cell with the net direction of flux determined by the electrochemical gradients of Na, K and Cl. In many cells, the inwardly directed Na gradient is the most prominent, and net movement of these ions is directed into the cell (Chipperfield, A., supra; Haas, M., supra; O'Grady, et al., *Am. J. Physiol.*, 253:C177–C192, 1987). Other characteristic features of Na—K—Cl cotransport include: 1) a high ion selectivity for Na, K and Cl; 2) an absolute requirement for the presence of all three ion species to operate; and 3) specific inhibition by "loop" diuretics (Palfrey, et al., supra).

There are two types of Na—K—Cl cotransporters with different electroneutral stoichiormetries. For most cells in which it has been studied (Ehrlich ascites tumor cells, rabbit kidney cells and duck red blood cells), the stoichiometry of cotransport is 1 Na^+:1K^+:2Cl^-, but in Squid axon the stoichiometry is different, 2 Na^+:1K^+:3Cl^-. Whereas the kinetic and pharmacological features of Na—K—Cl cotransport are quite constant among different cell types, the regulation of cotransport is heterogeneous. Elevation of intracellular cyclic AMP stimulates cotransport in some cells, while it inhibits cotransport in other cells. Similarly, elevation of cyclic GMP can have either stimulatory or inhibitory effects, and cotransport can be regulated by Ca and by phorbol esters, activators of protein kinase C (Chipperfield, A., supra; O'Donnell, et al., *Proc. Natl. Acad. Sci., USA*, 83:6132–6136, 1916; O'Donnell, et al., *Am. J. Physiol.*, 255:C169–C180, 1988; Grady, et al., supra). For instance, in cultured vascular endothelial cells, Na^+—K^+—2Cl^- cotransport is inhibited by elevations of intracellular cyclic AMP and cyclic GMP, and by activation of protein kinase C. In contrast, elevation of intracellular Ca stimulates endothelial cell Na^+—K^+—2Cl^- cotransport (O'Donnell, M. E., *Am. J. Physiol.*, 257:C36–C-44, 1989; O'Donnell, M. E., *J. Biol. Chem.*, 266:11559–11566, 1991). The reports of several studies have suggested that regulation of cotransport activity by vasoactive agonists and by extracellular tonicity involve a direct phosphorylation of the cotransporter (Lyle, et al., *J. Biol. Chem.*, 267:25428–25437, 1992; Pewitt, et al., supra; Torchia, et al., *J. Biol. Chem.*, 267:25444–25450, 1992).

Two primary physiological functions have been demonstrated for Na—K—Cl cotransport. The cotransporter participates in vectorial transport of ions across some epithelia, working in conjunction with the Na/K pump and other transport systems for Na, K and Cl (O'Grady, et al., supra). This function has been reported to occur in ciliary epithelial cells in the eye (Dong, et al., supra). The cotransporter also functions to regulate cell volume in a number of cell types, both epithelial and non-epithelial, in response to varying extracellular osmolarity (Eveloft, et al., *Am. J. Physiol.*, 252:F1–F10, 1987; Kobayashi, et al., supra, MacKnight, A. D. C., *Renal Physiol. Biochem.*, 3–5:114–141, 1988; O'Donnell, M. E., *Am. J. Physiol.*, 264:1316–1326, 1993; O'Grady, et al., supra). When cells are exposed to hypertonic media, they shrink rapidly as water exits the cell down its concentration gradient. In cells that utilize Na—K—Cl cotransport to regulate volume, the shrinkage of cells acti-

vates the cotransporter, which in turn mediates a net uptake of Na, K and Cl into the cell. As water re-enters the cell with the transported ions, the cell swells again. The Na—K—Cl cotransport system performs this function in a number of cell types, including vascular endothelial cells, avian erythrocytes, Ehrlich ascites tumor cells, human fibroblasts, chick cardiac cells, and cells of rabbit renal thick ascending limb (Kregenow, F. M., *Ann. Rev. Physiol.*, 43:493–505, 1981; MacKnight, A. D. C., supra; O'Donnell, M. E., supra, 1993). Trabecular meshwork and vascular endothelium both present highly regulated barriers to solute and water flux.

A regulatory volume increase can also be mediated in some cell types by combined actions of the Na/H exchange Cl/HCO_3 exchange systems (Kregenow, F. M., supra; MacKnight, A. D. C., supra). Exposure of cells to hypotonic media causes cells to swell rapidly as water enters the intracellular space, followed by a compensatory decrease in cell volume. The regulatory volume decrease appears to be mediated by a net efflux of ions through transporters separate from the Na—K—Cl cotransporter, for example the K and Cl conductive pathways (Eveloff, et al., supra; Kregenow, F. M., supra; MacKnight, A. D. C., supra). In addition, vasoactive agents have been shown to modulate the cell volume regulating system of avian erythrocytes. In these cells, the volume set point appears to be increased by catecholamines, such that the response of the Na—K—Cl cotransporter to extracellular tonicity is altered (Geck, et al., *J. Memb. Biol.*, 91:97–105, 1986).

The glaucomas comprise a heterogeneous group of eye diseases in which elevated IOP causes damage and atrophy of the optic nerve, resulting in vision loss. The underlying cause of the elevated IOP can be grossly divided into two pathophysiologic scenarios in which the drainage pathways are either physically closed off (as in the various forms of angle-closure glaucoma) or in which the drainage pathways appear anatomically normal but are physiologically dysfunctional (as in the various forms of open-angle glaucoma. Angle-closure glaucoma is nearly always a medical and/or surgical emergency, in which pharmacologic intervention is essential in controlling an acute attack, but in which the long-range management is usually surgical. Primary Open Angle Glaucoma (POAG), on the other hand, has a gradual, symptomless onset and is usually treated with chronic drug therapy. POAG is the most common form of glaucoma, comprising ≈80% of newly-diagnosed cases in the USA, and is the leading cause of blindness among African Americans.

Drugs currently used to treat glaucoma can be divided into those that reduce aqueous humor inflow and those that enhance aqueous humor outflow. The most commonly-prescribed drugs at present are the β-adrenergic antagonists, which reduce aqueous humor inflow through an unknown effect on the ciliary body. Other drugs that reduce aqueous inflow include inhibitors of carbonic anhydrase (e.g., acetazolamide and methazolamide) and the alpha-adrenergic agonist apraclonidine; both of these drug classes exert their clinical effects through a poorly-understood action on the ciliary body. Each of these drugs, although effective in many patients, is poorly tolerated in some because of profound and occasionally life-threatening systemic adverse effects.

Drugs that enhance aqueous humor outflow from the eye include miotics and the adrenergic agonists. The miotics exert a mechanical effect on the longitudinal muscle of the ciliary body and thus pull open the trabecular meshwork; they comprise both direct-acting parasympathomimetic agents (e.g., pilocarpine and carbachol) and indirect-acting parasympathomimetic agents (e.g., echothiopate). Miotic agents are highly effective in lowering IOP but have significant adverse effects, including chronic miosis, decreased visual acuity, painful accomodative spasm and risk of retinal detachment. Adrenergic agonists (e.g., epinephrine and dipivefrin) act on the uveoscleral outflow tract to enhance outflow through a mechanism that remains poorly understood. These drugs have perhaps the best safety profile of the compounds presently used to treat glaucoma but are among the least effective in their IOP-lowering effect.

Accordingly, the need exists for new and better methods of lowering intra-ocular pressure, particularly in the treatment of one of the leading causes of blindness, glaucoma.

SUMMARY OF THE INVENTION

The present invention provides a method for reducing intraocular pressure using a new class of compounds hitherto used as high ceiling diuretics, also known as loop diuretics, due to their ability to completely inhibit sodium chloride transport in the thick ascending limb of the loop of Henle. It has been discovered that the trabeculear meshwork cells of the mammalian eye regulate cell volume and fluid transport by means of a $Na^+—K^+—2Cl^-$ cotransporter mechanism, such as that found in the renal thick ascending limb of the loop of Henle. Compounds that substantially inhibit operation of this mechanism also increase the outflow of ocular fluids, thus lowering intraocular pressure. Such compounds are useful in treatment of diseases of the eye associated with elevated intraocular pressure, such as ocular hypertension and glaucoma.

Preferred compounds useful in the practice of this invention are furosemide, piretanide, benzmetanide, bumetanide, and torasemide, derivatives thereof with lipophilic and amphipathic characteristics designed to enhance penetration through the intact cornea, and pharmaceutically acceptable salts thereof. These outflow-increasing compounds are administered directly to the eye, either topically, by corneal iontophoresis, or by intracameral microinjection into the anterior chamber of the eye. The delivery of these compounds may be enhanced by the use of an erodible or sustained release ocular insert device. Lipophilic or amphipathic derivatives of these compounds are particularly preferred for topical administration.

A screening method is also provided to discover additional compounds with utility for lowering intraocular pressure by substantially inhibiting the $Na^+—K^+—2Cl^-$ cotransporter mechanism in trabecular meshwork cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a bar graph comparing the rates of potassium influx in bovine trabecular meshwork (TM) cells preincubated and assayed in Hepes-buffered medium containing no drugs (control) or ouabain, bumetamide, or a combination of ouabain and bumetanide. K^+ influx was assessed as ^{86}Rb influx (Rb quantitatively substitutes for K in this system) and the amount of radioactive contents was determined by liquid scintillation. Assay media were identical to preincubation media except that they also contained ^{86}Rb (1 μCi/ml). Data represent mean values±SEM of quadruplicate determination from six experiments.

FIG. 1B is a bar graph comparing bumetanide-sensitive potassium influx of bovine and human TM cells, evaluated in the presence or absence of ethacrynic acid (1 mM). Data represent means±SEM of quadruplicate determinations from six and two experiments (bovine and human TM cells, respectively).

FIG. 2A is a bar graph comparing the absolute cell volumes of bovine TM cells evaluated by electronic cell sizing following 30 minute exposure to bumetanide (0 or 10 μM) or ethacrynic acid (0 or 1 mM). Data are mean values±SEM, n=3.

FIG. 2B is a bar graph comparing the relative cell volumes of bovine TM cells as evaluated by radioisotopic determination of intracellular water space. Confluent trabecular meshwork cell monolayers were preincubated in media containing bumetanide (0 or 10 μM), ethacrynic acid (0 or 1 mM), 8-bromo-cyclic AMP (50 μM), 8-bromo-cyclic GMP (50 μM), or PMA (50 nM), and also containing either ^{14}C-urea or ^{14}C-sucrose. Specific activities of the radioactively tagged agents were used to calculate water space associated with total and residual extracellular radioactivty, respectively. Data are means±SEM, n=8.

FIG. 3A is a schematic diagram of cell culture chambers with filter inserts.

FIG. 3B is a graph comparing the effect of isotonic (300 mOsm) or hypertonic (400 mOsm) medium upon the relative cell volume of confluent bovine TM cells in isotonic (300 mOsm) or hypertonic (400 mOsm) medium. Mean cell volume was determined over the time course shown by electronic cell sizing with a Coulter Counter. Data are mean values±SEM, n=4.

FIG. 3C is a graph showing the effect upon bumetanide-sensitive K influx in bovine TM cell monolayers of changes in extracellular tonicity (mOsm) caused by addition of NaCl. Data are mean values±SEM, n=4.

FIG. 4 is a graph comparing the effects of isotonic medium, hypertonic medium, and bumetanide on permeability of trabecular meshwork cell monolayers grown on tissue culture filter inserts. The upper chamber also contained 1 μCi/ml ^{14}C-sucrose. Aliquots were removed from the lower chamber at times indicated and evaluated for radioactivity to quantitate sucrose. Data represent mean values±SEM of six replicates from a representative experiment.

FIG. 5A is a graph showing the effect over time upon intraocular pressure (IOP) of bumetanide versus vehicle (control) instilled into the eye of a rabbits (n=1)

FIG. 5B is a graph showing the effect over time upon IOP of bumetanide instilled into the eye of a second rabbit.

FIG. 6 is a bar graph comparing the average IOP-lowering effect of control and bumetanide calculated as the percent of baseline IOP.

FIG. 7 is a graph showing the percent reduction of intra-ocular pressure over time upon in vivo administration of torasemide.

A DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for increasing aqueous humor outflow in the eye of a human or other mammal by administration to the eye of an effective amount of a compound that substantially inhibits a Na^+—K^+—$2Cl^-$ cotransport mechanism, such as that found in the thick ascending limb of the loop of Henle. As used herein the term "Na—K—Cl transporter" refers generically to the cotransporter systems, but does not specify the particular stoichiometry of transport in the system described. On the other hand, as used herein the term Na^+—K^+—$2Cl^-$ cotransport system refers only to those cotransport systems having the indicated stoichiometry.

The output-increasing compound can be administered to the eye either topically or by intracameral microinjection into the anterior chamber of the eye, and is therapeutically useful in reducing the intraocular pressure in prevention and/or treatment of diseases characterized by elevated intraocular pressure, such as ocular hypertension and glaucoma.

It has been discovered that mammalian trabecular meshwork (TM) cells, i.e., bovine and human, possess a prominent Na^+—K^+—$2Cl^-$ cotransport system comparable in activity to that observed in vascular endothelial cells and the one found in the thick ascending limb of the loop of Henle. Using bovine TM cells, we have found that hormones and neurotransmitters shown by others to modulate outflow through the trabecular meshwork also alter the activity of the cotransporter in these cells. The results of the present study indicate that in TM cells, Na^+—K^+—$2Cl^-$ cotransport activity is stimulated by vasopressin, elevation of intracellular Ca and activation of protein kinase C, and that the cotransporter is inhibited by elevation of intracellular cyclic AMP or cyclic GMP. Thus, agents that elevate cyclic nucleotides and increase aqueous outflow also inhibit Na—K—Cl cotransport activity, whereas agents that elevate Ca or activate protein kinase C and decrease aqueous outflow also stimulate Na^+—K^+—$2Cl^-$ cotransport activity of TM cells.

The inventors herein have employed a variety of drugs to increase aqueous outflow and thereby decrease intraocular pressure. Although ethacrynic acid was known to increase aqueous outflow in both monkey and human eye, the mechanism of its operation was unknown. The inventors herein have discovered that ethacrynic acid and a class of related drugs heretofore known to be useful as diuretics achieve their therapeutic effect in the eye by inhibition of a previously undiscovered Na^+—K^+—$2Cl^-$ cotransport system in bovine and human TM cells.

The Na—K—Cl cotransport in TM cells is stimulated by elevation of extracellular tonicity. Thus, TM cotransport is increased by cell shrinkage, as would be predicted if the cotransporter mediates a regulatory volume increase in the TM cells. Exposure of TM cells to elevated extracellular tonicity has been found to cause an immediate cell shrinkage and a slower increase in cell volume that is blocked by bumetamide. These results indicate that TM cells do indeed regulate volume upon exposure to altered extracellular tonicity, and that they do so via a Na^+—K^+—$2Cl^-$ cotransport-dependent mechanism.

It has also been discovered that agonist- or drug-induced alteration of Na—K—Cl transporter activity in TM cells results in altered TM cell volume. Thus, agents, such as the phorbol ester PMA that stimulate the cotransporter also increase TM cell volume. By contrast, agents such as bumetanide, ethacrynic acid, cyclic AMP, and cyclic GMP that inhibit cotransport, decrease TM cell volume. The cotransporter system is shown to be important for maintenance of TM cell volume even in the absence of a tonicity challenge. Thus, cell shrinkage induces elevated cotransport activity, consistent with a role for the transporter in regulatory volume increase, but at the same time a change in cotransport activity drives a change in TM cell volume. This is a strong indicator that the Na—K—Cl cotransporter is of central importance for regulation of TM cell volume by hormones and pharmacologic agents.

Alteration of Na—K—Cl cotransport activity and/or TM cell volume modulates permeability of the trabecular meshwork. It has been discovered that flux of ^{14}C-sucrose across TM monolayers grown on filters was increased by exposure

of the cells to bumetanide in a manner that continued to increase over the 15 minute assay period, indicating that bumetanide-induced decrease in TM cell volume causes an increase in monolayer permeability. Exposure of the TM cells to hypertonicity, which causes cell shrinkage, also causes an increase in permeability. As with endothelial cells, the hypertonicity-induced permeability increase in TM cells appears to be transient. These findings indicate that the Na—K—Cl cotransporter and intracellular volume control are determinants of barrier function in cultured TM cell monolayers.

Of greatest importance for the invention herein, these findings indicate that Na^+—K^+—$2Cl^-$ cotransport-mediated volume regulation of trabecular meshwork cells plays an important, perhaps central, role in maintaining and regulating barrier function, and as a result, the homeostatic outflow of aqueous humor facility in the eye. Abnormal function or regulation of this system plays a role in the pathophysiology of ocular hypertension and glaucoma.

Abnormal function of the Na^+—K^+—$2Cl^-$ cotransporter in the eye can be modulated by administration directly to the eye of compounds that increase or decrease the biological activity of the Na—K—Cl cotransporter system in the TM cells. In treatment of ocular hypertension and glaucoma, wherein the pressure of the ocular fluid is undesirably elevated, compounds are administered directly to the eye that substantially inhibit activity of the Na^+—K^+—Cl^{-2} cotransporter, and hence increase outflow of aqueous humor from the eye.

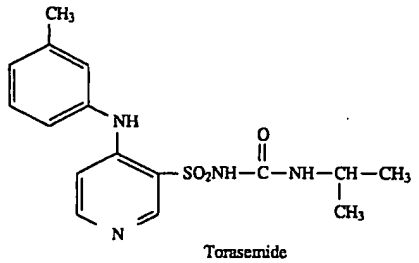
The in vivo biological activity of outflow-increasing compounds useful in the practice of this invention for increasing outflow of intraocular humor from the eye can be predicted using the well known in vitro test for determining compounds that inhibit the Na^+—K^+—Cl^{-2} co-transporter of rabbit renal thick ascending limb of the loop of Henle, as described by M. B. Burg et al. (*Am J. Physiol.* 210:1293–1298, 1966) as modified by R. Greger (*Pflüger's Arch.* 390:30–37, 1981) and R. Greger et al. (*Plfüger's Arch.* 389:175–176, 1981), both of which are incorporated herein by reference in their entireties. In brief, this in vitro test uses the Na+2Cl^-K^+ cotranspotter system found in the cortical thick ascending limbs of the loop of Henle of mammals, particularly rabbits, for example, female white New Zealand rabbits. The tubules are dissected and perfused according to the known techniques, but with the rate of perfusion through the lumen kept high (10–20 nl/min) to prevent changes in the ionic composition of the perfusate along the perfused tubule. As control perfusate, a solution containing (mM): NaCl 145; K_2HPO_4 1.6; KH_2PO_4 0.4; $MgCl_2$ 1; Ca gluconate 1.3 and glucose 5 is used on both sides of the epithelium with pH adjusted to 7.4

The transepithelial potential (V_{te}) is recorded continuously on both ends of the tubule. The specific transepithelial resistance (R_{te}) is calculated from the input resistance obtained by the injection of short current pulses (25–30 Na, 800 ms) into the tubule lumen. The ratio of both values (V_{te}/R_{te}) gives the equivalent short circuit current I_{te}. There is a direct relationship between the decrease of I_{te} and the inhibition of the $Na^+2Cl^-K^+$ co-transporter by contact with the test compound (R. Greger et al., *Klin. Wochenschr.* 61:1019–1027, 1983). For each drug under study, dose-response curves are established by adding compounds at various concentrations to the luminal perfusate with at least three determinations for each concentration. These response curves are then used to calculate the concentration necessary to block 50% of the short circuit current (IC_{50}). Compounds with therapeutic utility useful for increasing the outflow of aqueous humor from the eye, for instance in treatment of glaucoma, are characterized by having an IC50 at or below those of bumetanide or torasemide. To be useful in clinically enhancing aqueous humor outflow, compounds shown by this screening method to inhibit Na—K—Cl cotransport may also be screened for their activity in animal or human TM cells by testing their ability to inhibit Na—K—Cl cotransport in in vitro tissue culture. In this in vitro assay of pharmacologic activity, Na—K—Cl cotransport may be measured in these cells as ouabain-insensitive, bumetenide-sensitive potassium influx, using a radionuclide, such as ^{86}Rb, as a tracer for potassium. For usefulness in the practice of this invention, it is preferred that the outflow increasing composition inhibit ouabain-insensitive, bumetanide-sensitive potassium influx in human or bovine TM cells by at least 50%. Details of this method have been published previously in M. E. O'Donnell, *J. Biol. Chem.* 264:11559–11566, 1991, which is incorporated herein by reference in its entirety. An illustration of the use of this method is found in Example 1B of this application.

Compounds to be administered to the eye topically in the practice of this invention must not only inhibit Na^+—K^+—Cl^{-2} cotransporter system, but must also be sufficiently lipophilic to penetrate the corneal membrane. The lipophilicity of a compound is expressed in terms of an octanol:water coefficient, determined by the standard technique of radiolabelling the compound and introducing a small amount into equal volumes of octanol and tris buffer (50 mM, pH 7.4). Generally the lipophilicity (log P') is expressed as the logarithm of the partition coefficient in n-octanol/phosphate buffer, pH 7.4 using the well known shake-flask method as described by Cloux, et al., *J. Pharm. Belg.*, 43:141–151, 1973, which is incorporated herein by reference in its entirety. The coefficient of lipophilicity (log P') of the compounds useful for topical application to decrease intraocular pressure is preferably at least 0.005, and more preferably at least 0.01.

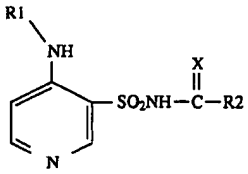
The lipophilicity of the aqueous humor outflow-increasing compounds of this invention can also be determined using a reversed phase, high performance liquid chromatograph (RP-HPLC) system for determination of the log P' of the drug as described in B. Masereel, et al. (*J. Pharm. Pharmacol.* 44:589–593, 1992), which is incorporated herein by reference in its entirety. Briefly, a reversed phase column (RP-18) is equilibrated with n-propanol/phosphate buffer, pH 7.4 at a ratio of 30:70). Compounds to be tested are dissolved and eluted with the same solution. A series of standards with a wide range of lipophilicity, as determined by the shake-flask method, is run and a calibration curve is established for each session. KNO_3 is injected to determine the void volume and log $k'=\log(t_r-t_o)/t_o$ is determined, wherein t_r is the drug retention time and t_o is the retention time of NO_3—. Calibration curves are calculated using log P' and log k' values. Log P' values of other compounds are obtained by interpolation of the standard curves.

Among the preferred outflow-increasing compounds of this invention are lipophilic derivatives of torasemide, and biologically compatible salts thereof, which are potent blockers of the $Na^+2Cl^-K^+$ co-transporter in the thick ascending limb of the loop of Henle (J. Delarge, *Arzneim. Forsch.* 38:144–150, 1988). These compounds combine a high degree of lipophilicity and biological activity. The chemical structure of torasemide (also known as Demodex™ and torsemide) is represented by the following formula:



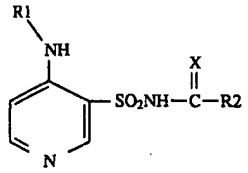
Torasemide

The chemical structures of the lipophilic derivatives of torasemide are represented by the following general formula:



wherein R^1 is selected from the group consisting of 3-methyl benzyl and 6–12 membered substituted or unsubstitued cycloalkyls, preferably an unsubstituted cyclo-octyl or a m-substituted phenyl, where the substituent is selected from $-CH_3$, $-CF_3$, or —Cl; and further wherein R^2 is selected from the group consisting of aminoisopropyl, 6–8 membered aminocycloalkyls and perhydroazepine. As shown by the data in Table 1 below, an increase of the number of methylenes of the R^1 cycloalyl group increases the inhibitory potency (compounds 9–11, 12–14, 15–17, and 21–23), whatever the size of R^2, but a cyclododecyl moiety in this position results in an inactive compound (compound 18). Incorporation of the distal nitrogen of the sulphonylurea moiety into a saturated ring leads to very active molecules (compounds 21–24). And a piperidine ring in the R^2 position (compound 22) increases inhibition potency compared with perhydroazepine (compound 24). Comparison of the log P' with the number of carbons in R^1+R^2 for each cycloalkyl disubstituted compound indicates that an increase of one methylene results in an enhanced lipophilicity of some 0.35 log units. Moreover, there is a correlation between log P' and inhibition potency.

TABLE 1



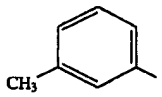
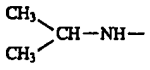
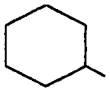
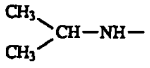
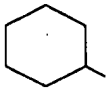
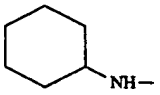
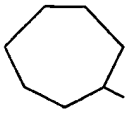
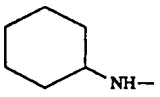
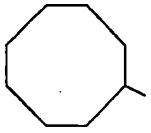
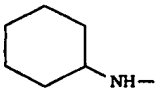
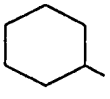
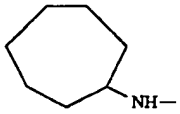
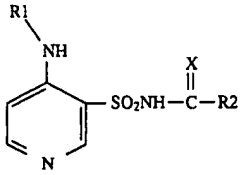
Compound	N° R1	R2	X	log P'	IC50
7			O	+0.45	0.30
8			O	+0.68	0.70
9			O	+1.33	19
10			O	+1.67	3.5
11			O	+2.06	0.47
12			O	+1.72	9.6

TABLE 1-continued



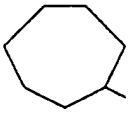
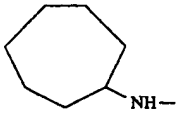
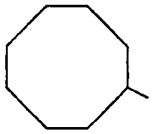
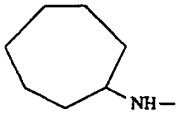
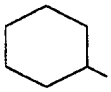
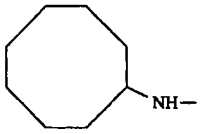
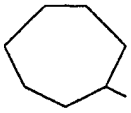
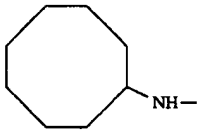
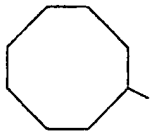
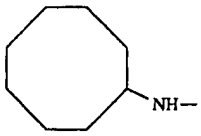
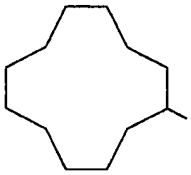
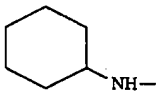
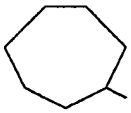
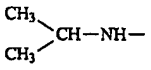
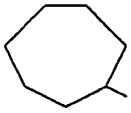
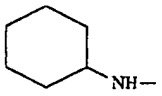
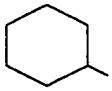
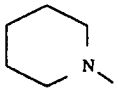
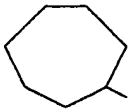
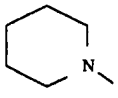
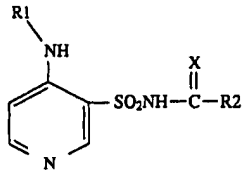
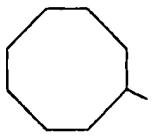
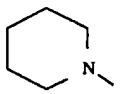
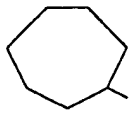
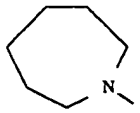
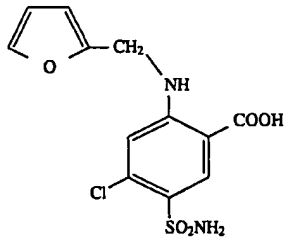
Compound	N° R1	R2	X	log P'	IC50
13			O	+2.06	2.8
14			O	+2.45	2.0
15			O	+2.07	14
16			O	+2.44	1.7
17			O	+2.70	0.56
18			O	+3.45	>100
19			S	+0.96	1.3
20			S	+1.79	0.79
21			O	+0.62	0.82
22			O	+1.24	0.25

TABLE 1-continued



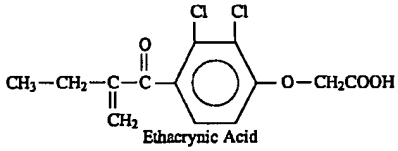
Compound	N° R1	R2	X	log P'	IC50
23			O	+1.65	0.15
24			O	+1.53	0.80
25				0.92	3.0

Methods of synthesis of torasemide and of the lipophilic torasemide derivatives of this invention are well known in the art, particularly as disclosed in B. Masereel et al., *J. Pharm. Pharmacol.* 44:589–593, 1992, which is incorporated herein in its entirety, anti in U.S. Pat. No. 4,861,786 to Demmer et al., which is incorporated herein in it entirety.

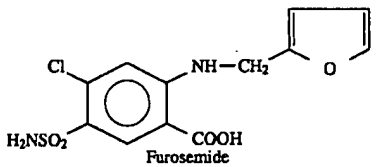
Additional compounds useful in the practice of this invention are a class of high ceiling diuretics, also known as loop diuretics, due to their ability to completely inhibit sodium chloride transport in the thick ascending limb of the loop of Henle at luminal concentrations of the drug in the range expected to occur in vivo. These drugs act at the luminal face of the epithelial cells to inhibit the Na^+—K^+—$2Cl^-$ cotransport mechanism (*Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 8th Ed., A. G. Gilman et al., Ed., Pergamon Press, New York, Elmsforth, N.Y., 1990, page 722).

Because these drugs inhibit the cotransport mechanism discovered by the inventors herein to operate in TM cells in the mammalian eye, all drugs of this class of diuretics are also useful for increasing outflow of aqueous humor from the eye when administered as disclosed herein. Hence, they are useful therapeutics for treatment of glaucoma and other conditions attributable to unnaturally high intraocular pressure. Three drugs of this class, whose chemical structures are shown in Table 2 below, are in clinical use in the United States as diuretics: ethacrynic acid, furosemide, and bumetanide. A number of other compounds in this class, including benzmetanide and piretanide, some of which are in used in other countries, are described in R. Greger and P. Wangemann, "Loop Diuretics," *Renal Physiology* 10:174–183, 1987, which is incorporated herein by reference in its entirety. It is also contemplated that derivatives of these compounds, as well as other compounds, can be used in the practice of this invention shown to inhibit the Na^+—K^+—$2Cl^-$ cotransport mechanism by the thick ascending limb screening test and the in vitro assay of cotransport activity and inhibition in cultured TM cells taught herein taught herein.

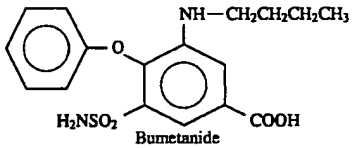
TABLE 2



Ethacrynic Acid



Furosemide



Bumetanide

In two extensive studies (E. Schlatter et al., *Pflügers Arch.* 405:367–376, 1985 and M. Wittner et al., *Pflügers Arch.* 408:54–62, 1987) the structure-activity relationships of

furosemide analogues and torasemide analogues have been examined, and general conclusions have been drawn regarding the minimum requirements needed for reversible interaction with the Na^+—K^+—$2Cl^-$ carrier. These requirements can be summarized as follows: (1) an anionic group is mandatory—this group can be a tetrazolate, a sulfonate, a carboxylate, or a sulfonyl urea; (2) in ortho or meta position to the anionic group, a secondary (e.g., furosemide) or a tertiary (e.g. piretanide) amine is needed; (3) this amino group links the anionic moiety to an apolar residue, and (4) in meta position to the anionic group, a sulfonamide group (e.g. piretanide) or a pyridino nitrogen (e.g., torasemide) is necessary (R. Weger, et al., supra, p. 179).

The outflow-increasing compounds disclosed herein, which inhibit the Na^+—K^+—$2Cl^-$ cotransport mechanism, can be administered either topically or by microinjection into the trabecular meshwork. For topical administration, the compound is dissolved in a pharmaceutically acceptable carrier substance, e.g., physiological saline. Additional pharmaceutically acceptable carrier substances can readily be supplied by one skilled in the art. For compounds having limited water solubility, the liquid carrier medium can contain an organic solvent, for example, 3% methyl cellulose. Methyl cellulose provides, by its high viscosity, increased contact time between the compound and the surface of the eye, and therefore increases corneal penetration. Corneal penetration can also be increased by administering the compound mixed with an agent that slightly disrupts the corneal membrane, for example 0.025% benzalkonium chloride, which also serves as a bacteriostatic preservative in various commercial formulations). Corneal penetration may also be increased by delivering a suspension of liposomes that incorporate the therapeutic compound, as described by Davies et al. ("Advanced Corneal Delivery Systems: Liposomes" in *Opthalmic Drug Delivery Systems*, A. K. Mitra, Ed., Vol. 58, pages 289–306 in the series *Drugs and the Pharmaceutical Sciences*, 1993, Marcel Dekker, Inc., New York. The outflow-increasing compound is administered periodically (for example, one time per week to ten times per day). Administration is by applying drops of the compound in solution using an eye dropper, such that an effective amount of the compound is delivered through the cornea to the trabecular meshwork. Administration may also be by a sustained-release formulation, such as a liposome, or via an ocular insert designed to enhance the dwelling time of the compound in the tear film and improve patient compliance with therapy, such as those described by R. Bawa, in A. K. Mitra, Ed., supra, Chapter 11, pages 223–260.

The "effective amount" of the compound to be delivered in one administration will depend on individual patient characteristics, e.g. the severity of the disease, as well as the characteristics of the administered compound, such as its lipophilicity and biological activity in stimulating the Na^+—K^+—Cl^{-2} cotransporter system in TM cells. Generally, an "effective amount" is that amount necessary to substantially inhibit the Na^+—K^+—Cl^{-2} cotransporter mechanism or establish homeostasis of the aqueous fluid in the eye as indicated by the intraocular pressure. Intraocular pressure reflects the balance between the production and outflow of aqueous humor, and the normal range is 2.09±0.33 kPa (15.8±2.5 mmHg) as measured by applanation tonometry (by planating the corneal surface) (*Harrison's Principles of Internal Medicine*, 13th Ed., Isselbacher et al., Ed., McGraw Hill, Inc., New York, p. 105). Typically, each drop contains 50–100 microliters of a 5–10 mM solution of the compound, so that 0.025 to 0.10 moles of the compound are delivered to each eye per day. Systemic absorption of the drug can be minimized by digital compression of the inner canthus of the eye during and for a short time following its instillation into the eye.

Direct microinjection of the solubilized compound to a site near the trabecular meshwork offers the advantage of concentrating the compound in the location where it is needed, while avoiding the possibility of side effects resulting from generalized exposure of the eye to the compound. Microinjection may also provide the advantage of permitting infrequent periodic administration, for example every few weeks, months, or even years, in contrast to the more frequent administrations required in the case of topical administration. Also, direct microinjection may promote the washing out of the trabecular meshwork of extracellular material interfering with fluid outflow. Preferably microinjection is administered via subconjunctival injection, most preferably into the superior aspect of the globe at the 12:00 o'clock position, from which point the drug reaches the intraocular space by diffusing passively across the scleral fibers, which offer essentially no barrier to penetration. Dosage for microinjection, like that for topical administration, varies with the above-mentioned parameters. Typically, microinjection dosage is such that a final concentration of the compound within the trabecular meshwork of 0.01 to 1.0 mM is reached.

The following examples illustrate the manner in which the invention can be practiced. It is understood, however, that the examples are for the purpose of illustration and the invention is not to be regarded as limited to any of the specific materials or conditions therein.

EXAMPLE 1

A. Cell Culture.

Bovine trabecular meshwork (TM) cells (Department of Ophthalmology, Lions of Illinois Eye Research Institute, Chicago, Ill.) and human trabecular meshwork isolated by methods based on those of Polansky et al. (*Invest. Ophthalmol. Vis. Sci.*, 18:1043–1049, 1979). Briefly, for isolation of bovine TM cells, eyes from healthy, freshly slaughtered young cows were enucleated. The TM was surgically excised, taking care not to include surrounding tissues. Explants were cut into small pieces (~1 mm^3), put in collagen-coated 175 cm^2 tissue culture flasks without medium for 1 minute until adhering, then growth medium was added to the flask. The media used was Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), essential and nonessential. amino acids, glutamine, and penicillin/streptomycin. The explants were maintained in a humidified CO_2 incubator at 37° C. and 5% CO_2. When cells growing out of the explant reached confluence, they were trypsinized and subcultured. Cultures that appeared to contain non-trabecular meshwork cells were discarded. Cultures were maintained by refeeding every 2 days and splitting weekly.

Similar techniques were used in isolation of human TM cells, except that human TM derived from three sources: 1) research donor eyes (presumed to be normal) from the eye bank of the University of California, Davis Medical Center; 2) otherwise healthy eyes enucleated because of life-threatening malignancies in the posterior pole (e.g., retinoblastoma or choroidal melanoma); and 3) trabeculectomy specimens. At the time of trabeculectomy surgery, the surgeon created a partial thickness scleral flap, unroofing the TM at the surgical limbus. A small piece (≈1 to 2 mm^3) was then excised to create the surgical fistula.

Both types of TM cells were maintained in collagen-coated tissue culture flasks and were used between passages 8 and 12 for bovine and between passages 3 and 8 for human. For experiments, cells were removed from the flasks by brief typsinization and were subcultured onto 24 well plates coated with collagen Type I (Collaborative Research, Inc., Bedford, Mass.) for radioisotopic transport and cell volume experiments or onto collagen-coated tissue culture filter inserts (Biocoat™, 13 mm diameter, 0.45 μm pore size (Collaborative Research Inc.). Cells were used 5–7 days later as confluent monolayers and growth medium was replaced every 2 days.

B. Transport Measurements.

Agents known to increase aqueous outflow should inhibit activity of the cotransporter, and agents which decrease aqueous outflow should stimulate the cotransporter. Further these agents should alter Na^+—K^+—$2Cl^-$ cotransport with a potency similar to that observed for their actions on trabecular meshwork function.

Na—K—Cl cotransport was measured as ouabain-insensitive, bumetanide-sensitive potassium influx, using ^{86}Rb as a tracer for potassium. Details of this method have been published previously (O'Donnell, M. E., supra, 1989). Briefly, bovine or human TM cell monolayers on 24 well plates were equilibrated for 10 minutes at 37° C. in a Hepes-buffered minimal essential medium (MEM) containing (in mM): 144 Na, 147 Cl, 5.8 K, 1.2 Ca, 4.2 HCO_3, 0.4 HPO_4, 0.4 H_2PO_4, 0.4 SO_4, 5.6 glucose and 20 Hepes. The cells were then preincubated and assayed for 5 minutes each with Hepes-buffered MEM containing 1 or 0 mM ouabain (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), 10 or 0 μM bumetanide (Hoffman-LaRoche, Nutley, N.J.) and either 145 or 0 mM Na and Cl (Na isosmotically replaced by choline, Cl isosmotically replaced by gluconate). The assay medium also contained ^{86}Rb (1 μCi/ml) (Dupont New England Nuclear, Boston, Mass.). The assay was terminated by rinsing the wells with ice-cold isotonic $MgCl_2$, then extracting the contents with 0.2% sodium dodecyl sulfate (SDS), and determining the amount of radioactive contents by liquid scintillation. Osmolarities of all preincubation and assay media were verified by osmometry (Model 3W2, Advanced Instruments, Norwood, Mass.).

Evaluation of K influx in cultured trabecular meshwork cells revealed the presence of a robust Na^+—K^+—$2Cl^-$ cotransport system. As shown in FIGS. 1A and 1B, bovine TM cells exhibited a total K uptake of 49±1.47 μmol K/gm protein/min. The K influx was decreased by 10 μM bumetanide to 22.09±1.14 μmol K/gm protein/min. and by 1 mM ouabain to 24.16±1.36 μmol K/gm protein/min, indicating the presence of both Na/K pump activity and Na^+—K^+—Cl^{-2} cotransport activity. A bumetanide-sensitive K influx was also observed in the presence of ouabain. Thus, high levels of bumetanide-sensitive K influx are present in these cells, whether measured with or without Na/K pump inhibition, namely 20.1 and 26.9 μmol K/gm protein/min, respectively.

Removal of either Na or Cl from the assay media abolished bumetanide-sensitive K influx of the trabecular meshwork cells, indicating an obligate presence of Na and Cl for the bumetanide-sensitive K influx to occur, as is required in Na—K—Cl cotnansport activity.

In the bovine TM cells, cotransport-mediated K influx was approximately 55% of the total influx, compared to 50% of Na/K pump-mediated K influx. This result shows that the cotransporter provides a substantial contribution to TM cell function. Half-maximal inhibition of K influx was observed with 0.1 μM bumetanide, whether in the presence or absence of ouabain. This potency is similar to that reported for a number of other cell types, including vascular endothelial cells (Chipperfield, A., supra; O'Donnell, M. E., supra, 1989; O'Grady, et al., supra). As shown in FIG. 1B, high magnitude bumetanide-sensitive K influxes were observed in human as well as in bovine TM cells.

Previous studies of other cell types have shown that ethacrynic acid, structurally distinct from bumetanide, also inhibits Na^+—K^+—$2Cl^-$ cotransport activity (Palfrey, et al., supra). If exposure of TM cells to ethacrynic acid inhibits Na^+—K^+—$2Cl^-$ cotransport activity, then it should decrease or abolish the bumetanide-sensitive portion of K influx in these cells. As shown by the results summarized in FIG. 1B, bumetanide-sensitive K influx of both bovine and human TM cells was abolished in the presence of 1 mM ethacrynic acid.

In order to determine whether activity of the cotransporter is responsible for mediating net ion uptake, it is necessary to evaluate the influence of cotransport activity on cell ion content. Thus, studies were conducted to determine the magnitude of net K uptake mediated by the TM cell Na^+—K^+—$2Cl^-$ cotransporter. For determination oft he net potassium uptake, the potassium contents of TM cells were evaluated by atomic absorption spectrophotometry, as described previously by O'Donnell, M. E. (supra, 1993). Briefly, cells were preincubated in 10 μM bumetanide for 5 minutes, and assayed as described above, with the exception that no ^{86}Rb was present during the assay. Cell ionic contents were extracted with 5% trichloroacetic acid following removal of extracellular potassium by washing the monolayers with ice-cold 0.1M $MgCl_2$.

Under steady state conditions, Na^+—K^+—$2Cl^-$ cotransport mediated a net uptake of K such that inhibition of the cotransporter results in a decrease in cell K (via other transport pathways). This bumetanide-sensitive net K uptake was found to be 7.78±1.98 μmol K/gm protein/min. If either Na or Cl was omitted from the incubation medium, the, bumetanide-sensitive net K uptake was abolished (1.71±2.49 and −2.62±2.82 μmol K/gm protein/min, respectively, n=8).

C. Measurements of Cell Volume.

If Na—K—Cl cotransport of TM cells is important for regulation and maintenance of cell volume, then alteration of cotransport activity should lead to altered intracellular cell volume. Omission of either Na, K or Cl from the assay medium should also cause a decrease in cell volume because the Na—K—Cl cotransporter cannot operate if any one of the transported ion species is absent from the extracellular medium. In addition, second messengers (e.g., cyclic AMP and cyclic GMP), as well as hormones and neurotransmitters found to be inhibitory to cotransport should cause a decrease in trabecular meshwork cell volume. Similarly, second messengers (e.g., Ca and protein kinase C) identified to be stimulatory to cotransport activity should cause an increase in trabecular meshwork cell volume, as should hormones and neurotransmitters identified to stimulate the cotransporter. Further, if Na—K—Cl cotransport is responsible for mediating hormone-, neurotransmitter- and second messenger-induced increases in cell volume, then blocking cotransport activity by bumetanide should prevent the elevation of cell volume induced by these agents. In addition, the potency with which each of these agents alters Na—K—Cl cotransport activity should be similar to the potency for alteration of intracellular volume.

The intracellular volume of human and bovine TM cells was evaluated by two methods: 1)radioisotopic evaluation of TM monolayer intracellular water space using ^{14}C-urea and

^{14}C-sucrose as markers of total and extracellular space, respectively; and 2) electronic cell sizing of suspended TM cells, using a Coulter Counter™ assay (Coulter Electronics, Ltd., Hialeah, Fla.). Details of these methods have been described previously by O'Donnell (O'Donnell, M. E., supra, 1993).

By the first method, cell monolayers were preequilibrated for 30 minutes in Hepes MEM at 37° C. in an air atmosphere, then incubated for 20 minutes in Hepes MEM containing 0 or 10 μM bumetanide, ethacrynic acid, or other agents to be tested, and finally incubated for 10 minutes in the same medium containing either ^{14}C-urea or ^{14}C-sucrose (both at 1 μCi/ml). Monolayers were then rinsed with isotonic ice-cold $MgCl_2$ and radioactivity of SDS extracts determined by liquid scintillation. Specific activities (counts per minute/ml) of ^{14}C-urea and ^{14}C-sucrose in the assay medium were determined and used to calculate water space associated with trapped radioactivity (expressed as μl/mg protein. Intracellular volume was calculated as the difference between the water space determined for ^{14}C-urea (a marker for intracellular plus trapped extracellular space) and ^{14}C-sucrose (a marker for trapped extracellular space). FIG. 2A shows that when the volume of the cells was assessed by electronic cell sizing of cell suspensions, exposure of the cells to either bumetanide or ethacrynic acid caused significant reduction of intracellular volume as compared to exposure to the Hepes MEM control.

By the second method, TM cells were trypsinized briefly in Ca-free medium, then rinsed with medium containing trypsin inhibitor and suspended in Hepes MEM. Mean cell volumes were then analyzed by electronic cell sizing on a Coulter Counter™ radioassay (Model ZM). with channelizer (C256), using at least 50,000 cells per data point and an orifice diameter of 140 μm. Aliquots of suspended cells were diluted into Hepes MEM containing the tonicity and/or agents to be evaluated. Mean cell volumes of each suspension aliquot were followed over time, starting at 1 minute after addition of cells to the assay media. Cell suspensions were maintained at 37° C. throughout the assay period. Absolute volumes (picoliters/cell) were calculated from distribution curves of cell diameter, using a standard curve generated by polystyrene latex beads of known diameter.

If hormones and neurotransmitters alter activity of Na^+—K^+—$2Cl^-$ cotransport, second messengers reported to be elevated in trabecular meshwork cells by these agents should also modulate activity of the cotransporter. Thus, experiments were conducted to evaluate the effect on activity of the cotransporter of altering intracellular second messenger levels The effects of 20 minute exposures of cyclic AMP, cyclic GMP and the phorbol ester PMA on TM cell volume are also shown in FIG. 2B. Consistent with their effects on Na^+—K^+—$2Cl^-$ cotransport activity, both cyclic nucleotides reduced TM cell volume while the phorbol ester PMA increased cell volume. These findings are consistent with the hypothesis that alteration of Na^+—K^+—$2Cl^-$ cotransport activity by hormones, neurotransmitters and drugs can cause alteration of TM cell volume.

D. Permeability Measurements.

A role for Na^+—K^+—$2Cl^-$ cotransport in regulation of trabecular meshwork cell volume requires that changes in activity of the cotransporter result in net changes in cell content of Na, K and/or Cl. If the cotransporter is involved in maintaining intracellular volume under steady state conditions, then inhibiting activity of the cotransporter should cause a decrease in Na, K and/or Cl content of the cells, which would then cause a loss of water from the cells and shrinkage.

To investigate the possibility that alteration of TM intracellular volume results in modulation of trabecular meshwork barrier permeability, we evaluated the flux of ^{14}C-sucrose across confluent TM cell monolayers grown on permeable filters. Cells were grown to confluence on collagen-coated dual level tissue culture filter inserts placed in covered multiwell cluster plates, as shown schematically in FIG. 3A, wherein lower chamber 2 is formed by suspension of membrane 4 with cells attached into holder 6, which is covered by cover 8. Upper chamber 10 is formed by the space within the holder above the membrane. Growth media (EMEM) volumes of 0.5 and 1.5 ml were used for upper and lower chambers, respectively, as previously described (Kajimura, et al., *FASEB J.*, 8:A1045, 1994) for evaluation of endothelial cell monolayers and for evaluation of water permeability of TM cell monolayers (Perkins, et al., *Invest. Ophthalmol. Vis. Sci.*, 29:1836–1846, 1988). One insert was placed in each of 12 wells in a cluster plate. Permeability was assessed as the flux of ^{14}C-sucrose across the TM cell monolayers. Both upper and lower chambers of the multiwell cluster plates were equilibrated for 30 minutes in Hepes MEM at 37° C. in a gyrotary water bath, shaking at 60 cycles per minute, to ensure adequate mixing. Aliquots of medium were sampled at various times from the lower chamber to determine sucrose content. The sample volume removed was replaced each time with an equal volume of isotonic or hypertonic sucrose-free medium. Sucrose content of the samples was determined by spectrofluorometric methods. The amount of solute was calculated from the radioactivity present in the sample and the specific activity (cpm/μmol) of the ^{14} C-sucrose.

As shown in FIG. 4, shrinkage of TM cells by exposure to hypertonic medium caused an elevation of monolayer permeability to sucrose above that observed with isotonic medium. Permeability was also increased by bumetanide, with the rate of sucrose accumulation increasing over the 15 minute assay period, consistent with our finding that bumetamide causes reduction of TM cell volume.

E. Statistical Analysis.

Experimental results were analyzed by two-way analysis of variance, using Bonferroni-Dunn post-hoc ANOVA (Statview, Abacus Concepts, Inc., Berkeley, Calif.). The criterion for significance in all cases was a p value$\geqq$0.05.

EXAMPLE 2

Regulation of trabecular meshwork cell Na^+—K^+—$2Cl^-$ cotransport activity.

The hypothesis that hormones and neurotransmitters can modulate trabecular meshwork cell volume by altering Na^+—K^+—$2Cl^-$ cotransport activity requires that these agents alter the net uptake of Na, K and/or Cl occurring via the cotransporter. The effects of hormones, neurotransmitters and second messengers on Bumetanide-sensitive K influx of bovine trabecular meshwork cells were assessed using the procedures of Example 1. The cells were preincubated and assayed in the presence of vasopressin (100 nM) (Peninsula Laboratories, Belmont, Calif.); norepinephrine (NE) (10 μM) (Sigma Chemical, ST. Louis, Mo.); acetylcholine (Ach) (1 μM) (Sigma), 8-bromo-cyclic AMP (cAMP) (50 μM) (Sigma); 8-bromo-cyclic GMP (cGMP, 50 μM) (Sigma); A23187 (1 μM) (Sigma); phorbol 12-myristate 13-acetate (PMA) (10 nM) (Sigma); or Ca-free medium plus 5 μM 1,2-bis(o-anfinophenoxy)ethane-N,N,N',N-tetraacetic acid acetoxymethyl ester (BAPTA-AM), following a 30 minute pre-exposure of the cells to the membrane permeant Ca chelator BAPTA-AM. The control is the Hepes-buffered

medium containing Na, K, Cl, CA and other ions as described in Example 1B. In Table 3 below the results of these studies are shown as μmol/g protein/min. The data represent the mean values±SEM of quadruplicate determinations from two experiments.

TABLE 3

BUMETANIDE-SENSITIVE K INFLUX (μmol/g protein/min)

Control	25.6 ± 0.71	NE	18.6 ± 1.79
VP	40.4 ± 1.05	Ach	20.2 ± 1.26
A23187	33.4 ± 5.33	cAMP	19.6 ± 0.96
PMA	34.0 ± 1.33	cGMP	19.7 ± 0.74
Ca-free	4.9 ± 1.06		

The data in Table 3 show that exposure of the cells to the divalent cation ionophore A23187 at a concentration of 1 μM to elevate intracellular Ca^{2+} caused stimulation of Na—K—Cl cotransport activity. When the cells were pre-incubated for 30 minutes with BAPTA-AM at a concentration of 5μM, and subsequently assayed in the presence of BAPTA-AM and Ca-free medium, activity of the Na^+—K^+—$2Cl^-$ cotransporter was markedly reduced. Activation of protein kinase C by assaying the cells in the presence of the phorbol ester phorbol 12-myristate 13-acetate also stimulated cotransport in these cells. Exposure of the cells to vasopressin also stimulated cotransport activity. In contrast, exposure of the cells to the permeable cyclic nucleotides 8-Br-cyclic AMP (50 μM) or 8-Br-cyclic GMP (50 μM) caused inhibition of bumetanide-sensitive K influx, as did exposure to either norepinephrine or acetylcholine.

EXAMPLE 3

Effect of extracellular hypertonicity on trabecular meshwork cell volume and Na^+—K^+—$2Cl^-$ cotransport activity.

The hypothesis that the Na—K—Cl cotransport system of trabecular meshwork cells participates in restoration of cell volume following hypertonicity-induced cell shrinkage by mediating a compensatory re-swelling of the cells requires that the cotransporter be stimulated by cell shrinkage. Also, extracellular tonicity should be directly related to cotransport activity, with the greatest sensitivity occurring within a physiological range (i.e., approximately 270–330 mOsm). That is, the largest changes in cotransport activity should be elicited by relatively small changes in osmolarity in the range of 300 mOsm (rather than at 400 mOsm, for example). A role for Na—K—Cl cotransport in mediating cell shrinkage-induced regulatory volume increase also requires that cell shrinkage cause an increase in cell Na, K and Cl content by stimulating net uptake of these ions by the cotransporter following exposure of the cells to hypertonicity in the presence and absence of bumetanide.

In order to determine whether the Na—K—Cl cotransporter of TM cells may play a role in restoration of intracellular volume following hypertonicity-induced cell shrinkage, we evaluated the effect of hypertonicity on TM cell volume and Na^+—K^+—$2Cl^-$ cotransport activity. As shown in FIG. 3, exposure of the cells to hypertonic medium caused an immediate shrinkage of cells (as determined by Coulter Counter) to approximately 70% of control isotonic volume (at 1 minute, the earliest measurement). The intracellular volume of the cells then increased over a slower time course, with volume recovering to approximately 84% by 33 minutes as shown in FIG. 3A.

When the hypertonic medium contained bumetanide, however, the volume recovery, was greatly attenuated. This suggests that the cotransporter of TM cells contributes to restoration of intracellular volume following hypertonicity-induced cell shrinkage. Consistent with the theory, as shown in FIG. 3B, we found that exposure of the TM cells to hypertonicity also caused stimulation of Na^+—K^+—$2Cl^-$ cotransport activity. Maximal stimulation by hypertonicity was observed between 350 and 400 mOsm.

EXAMPLE 4

Male albino New Zealand rabbits weighing≧2 Kg were used for in vivo experiments performed under various active animal use protocols approved by the Institutional Animal Care and Use Committee at UC Davis. Animals were sedated with an intravenous dose of Napentabarbitol, 1–2 mg/Kg to permit their handling and frequent measurement of intraocular pressure (IOP). Topical anesthesia consisting of Proparacaine HCl (Alcaine™) was used for IOP measurements. A pneumatonometer (Mentor O & O Classic Model 30 Penumatonometer™, Norwell, Mass.) calibrated to a known (25 mmHg) pressure prior to each experiment was used for all IOP measurements. This model of Pneumatonometer provides a readout of standard deviation of each measurement; only measurements with a standard deviation of ≦1 mmHg were accepted and most were less than 0.5 mmHg. Following sedation, IOP measurements were carried out over at least 30 minutes to assure that a stable baseline IOP was present.

Drugs and/or vehicle was administered to one eye of each animal via subconjunctival injection in order to bypass any barriers to drug penetration, such as an intact corneal and conjunctival epithelium and to achieve rapid and high concentrations of drug, bypassing epithelial barriers. The contralateral eye treated only with vehicle served as a control for each animal. Two syringes were prepared for each experiment, one containing drug and vehicle, the other containing only vehicle. Using topical anesthetic, 0.1 cc from each syringe was injected subconjunctivally into the superior aspect of the globe at the 12:00 o'clock position. Injections raised a prominent bleb, confirming subconjunctival placement of the drug or vehicle. This bleb then smoothed out over about ten minutes, indicating diffusion of the drug or vehicle throughout the subconjunctival space.

Measurements of intraocular pressure were taken at various intervals following the subconjunctival administration of the drug and/or vehicle. IOP in both eyes fluctuated over time reflecting the level of anesthesia. During the course of the experiment, the animal handlers and investigators were masked as to which eye had received the drug or control injection.

Two animals were treated with bumetanide prepared from the powder form to produce a concentration of 10^{-5}M, in a vehicle of DMSO. The control was treated with DMSO alone. Subconjunctival injections with a total volume of 0.1 cc were given to each eye. As shown by the data summarized in FIG. 5A, in the first animal treated with drug the effect of bumetanide on IOP was rapid and pronounced and lasted beyond two hours. As shown in FIG. 5B, in the second animal treated with drug, the average drop in IOP is even more pronounced. Data are expressed as a %-lowering of IOP relative to the contralateral, control eye. The FDA requires a 20% lowering of IOP for a drug to be considered clinically effective in the treatment of glaucoma and ocular hypertension.

FIG. 6 compare the average of two experiments (2 animals tested with bumetanide, 2 tested with torasemide) and reveals an average IOP lowering effect of over 20%.

EXAMPLE 5

Using the protocol described in Example 4 above, 0.1 cc of 1 μM torasemide (Burroughs Wellcome Co., Greenville, N.C. for Boehringer-Mannheim Pharmaceuticals, Rockville, Md.) diluted from the commercially-available 29 mM stock intravenous form with phosphate-buffered saline. The commercial vehicle contained polyethylene glycol (PEG) and Tris in unknown amounts. Following the rather large dilution, the concentrations of Tris and PEG would have been negligible. Therefore the phosphate buffered saline was used as the control vehicle. As can be seen by the results summarized in FIG. 7, torasemide produced an IOP-lowering effect with a time course and magnitude similar to that seen with bumetanide.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

We claim:

1. A method for increasing aqueous humor outflow in the eye of a mammalian patient to reduce the intraocular pressure therein, said method comprising administering to said eye a composition comprising an effective amount of a compound that inhibits the Na^+—K^+—$2Cl^-$ co-transport in the trabecular meshwork of the eye, or a pharamaceutically acceptable salt thereof, in a pharamacutically acceptable carrier; wherein the compound is bumetanide.

2. The method of claim 1 wherein the composition is administered by microinjection.

3. The method of claim 1 wherein the composition is administered topically.

4. The method of claim 3 wherein the composition further comprises a compound that enhances corneal penetration.

5. The method of claim 3 wherein the composition further comprises 0.025% benzalkonium chloride.

6. The method of claim 3 wherein the compound has an octanol:water coefficient of at least 0.005.

7. The method of claim 3 wherein the compound has an octanol:water coefficient of at least 0.01.

8. The method of claim 1 wherein the patient is human and administration is in treatment of glaucoma.

9. The method of claim 8 wherein dosage of the compound is 50–100 microliters of a 5–10 mM solution of the compound per day.

10. The method of claim 6 wherein dosage of the compound is 50–100 microliters of a 5–10 mM solution of the compound per day.

11. A method for screening compositions for utility in lowering intraocular pressure comprising:

a. determining the IC50 concentration of a composition containing bumetanide in the cortical thick ascending limb of the loop of Henle;

b. determining the inhibition of cotransport in mammalian trabecular meshwork cells; and

c. selecting the compositions with an IC50 at or below 0.30 and an inhibition of Na^+—K^+—$2Cl^-$ cotransport of at least 50%.

12. The method of claim 11 wherein the cortical thick ascending limb is in rabbit and the trabecular meshwork cells are bovine or human.

13. A method for screening compositions containing bumetanide for utility in lowering intraocular pressure comprising screening the compositions in a Na^+—K^+—$2Cl^-$ cotransporter mechanism screen, and selecting a composition that substantially inhibits a Na^+—K^+—$2Cl^-$ cotransporter mechanism.

14. The method of claim 13 wherein the Na^+—K^+—$2Cl^-$ cotransporter mechanism is in mammalian trabecular meshwork cells and the composition selected has an IC50 of about 0.30 or less.

15. The method of claim 14 wherein the trebecular meshwork cells are human.

16. The method of claim 14 wherein the trebecular meshwork cell are bovine.

17. The method of claim 14 wherein the composition selected further has a coefficient of lipophilicity (log P') of at least 0.005.

18. The method of claim 14 wherein the composition selected further has a coefficient of lipophilicity (log P') of at least 0.01.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,585,401

DATED : December 17, 1996

INVENTOR(S) : James D. Brandt
Martha E. O'Donnell
Fitz-Roy E. Curry

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, item [73],

Assignee: The Reents of the University of California, Oakland, California

is in error and should be as follows:

Assignee: The Regents of the University of California, Oakland, California

Signed and Sealed this

Fourth Day of March, 1997

Bruce Lehman

BRUCE LEHMAN

Commissioner of Patents and Trademarks

Attest:

Attesting Officer

THE JOURNAL OF EXPERIMENTAL ZOOLOGY 279:498–503 (1997)

Potential Contribution of Epithelial Na^+ Channel to Net Secretion of Aqueous Humor

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ABSTRACT The aqueous humor of the eye is secreted by the bilayered ciliary epithelium, consisting of the pigmented (PE) cell layer facing the stroma and the nonpigmented (NPE) cell layer facing the aqueous humor. Cells within each layer and between the two layers are linked by gap junctions, forming a ciliary epithelial syncytium. Unidirectional secretion from the stroma to the aqueous proceeds both through the cells (the transcellular pathway) and between the cells (the paracellular pathway). Net formation of aqueous humor must, however, be the algebraic sum of unidirectional secretion and unidirectional reabsorption from the aqueous humor back into the stoma. The mechanisms potentially underlying reabsorption of aqueous humor by the NPE cells have recently been addressed by studying the regulatory response (RVI) of anisosmotically shrunken NPE cells. The results indicated that epithelial Na^+ channels with a high affinity to amiloride likely contribute to reabsorption of solute from the aqueous humor. We have substantiated this possibility by using Northern analysis to identify in human ciliary body RNA a 3.7-kb transcript corresponding to the α-subunit of the amiloride-sensitive, αβγ-ENaC epithelial sodium channel. We have also found that the Na^+-channel inhibitor benzamil inhibits the RVI without affecting the cell volume of isotonic cell suspensions. This observation supports the hypothesis that the low conductance, highly selective epithelial Na^+ channel is activated by shrinkage and contributes to unidirectional reabsorption as aqueous humor. Examples are provided of how the integrative regulation of aqueous humor formation can involve conjugate actions on both unidirectional secretion and reabsorption. *J. Exp. Zool. 279:498–503, 1997.* © 1997 Wiley-Liss, Inc.

STRUCTURE OF THE CILIARY EPITHELIUM

The aqueous humor of the eye is secreted by the ciliary epithelium, a bilayered structure covering the surface of the ciliary body. The outer cell layer is formed by the pigmented ciliary epithelial (PE) cells, whose basolateral membranes face the ciliary body stroma. The inner cell layer consists of the nonpigmented ciliary epithelial (NPE) cells, whose basolateral surfaces face the aqueous humor. Only the NPE cells form tight junctions in vivo (Raviola and Raviola, '78). Low-resistance gap junctions connect cells within each layer and between the two layers, creating a functional syncytium (Raviola and Raviola, '78; Green et al., '85; Coca-Prados et al., '92; Edelman et al., '94; Oh et al., '94; Bowler et al., '96).

OVERVIEW OF CILIARY EPITHELIAL SECRETION

As in the case of transepithelial transport generally, fluid is translocated by the primary transfer of solute from the stroma to the aqueous humor, with water passively following. The slightly negative transepithelial potential (of ~1 mV) provides a driving force for Na^+ and other cations to move through the paracellular pathway (between the cells). However, the dominant contribution to unidirectional secretion of solute is likely to proceed by the transcellular pathway (through the cellular syncytium; Krupin and Civan, '95). Transcellular

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transfer from the stroma to the contralateral surface must involve at least three steps, each of which could theoretically be the rate-limiting process for aqueous humor formation. First, solute is taken up from the stroma by the PE cells; this step has been thought to be largely performed by a Na^+-K^+-$2Cl^-$ symport (Krupin and Civan, '95), but possibly also by Cl^-/HCO_3^- antiport exchange (Wiederholt et al., '91). Second, solute and water diffuse from the PE to the NPE cells through the gap junctions. Third, Na^+, K^+, and Cl^- are released by the NPE cells into the aqueous humor through the Na^+/K^+ exchange pump, K^+ channels, and Cl^- channels, respectively.

Recent results obtained by electron probe X-ray microanalysis of intact rabbit ciliary epithelium (Bowler et al., '96) bear on the issue of the rate-limiting site of aqueous humor formation. As summarized in Table 1, the elemental Cl concentration (in mmol/kg intracellular water) is some four-to-five times higher in the PE cells than the equilibrium distribution predicted at a membrane potential of ~–70 mV (Carré et al., '92). This datum indicates that the PE cells can accumulate solute against an electrochemical gradient under baseline conditions. The data also document that the intracellular elemental concentrations of Na, K, and Cl are approximately equal in the PE and NPE cells. This observation indicates that the gap junctions are also unlikely to limit the flow of solute from stroma to aqueous humor; otherwise, we would expect to find gradients in concentration between the PE and NPE cells. By exclusion, we conclude that the third step, the final net release of solute from the NPE cells into the aqueous humor is likely to limit the rate of ciliary epithelial secretion.

The net translocation of solute from the NPE cells into the aqueous must equal the difference between unidirectional secretion out of the cells and unidirectional reabsorption back into the cells. These two unidirectional fluxes appear to reflect the operation of very different transport mechanisms. In contrast to the Na^+/K^+ exchange pump, K^+ channels and Cl^- channels subserving unidirectional secretion, four different mechanisms (considered below) seem to underlie unidirectional reabsorption. Recent volumetric measurements of human NPE cells have suggested that one of the mechanisms underlying unidirectional reabsorption is an amiloride-sensitive epithelial Na^+ channel (Civan et al.,'96). These measurements were conducted with the simplest possible experimental model of aqueous humor reabsorption, the regulatory volume increase (RVI) of NPE cells (Civan et al.,'96). The human (ODM) NPE cells are first suspended in 50% hypotonic solution, producing swelling and a secondary release of KCl and water. Isotonicity is then restored by addition of sucrose 25 min after the initial suspension. The cells shrink to ~80% of their isotonic volumes, but then exhibit a secondary regulatory response (the RVI) in which solute and water are slowly taken up at 0.144 ± 0.007%/min (Civan et al.,'96). The RVI was significantly inhibited by amiloride at 20 μM, dimethylamiloride at 10 μM, and benzamil at 1 μM; the effects of amiloride and dimethylamiloride were not significant at 1 μM (Civan et al.,'96). Benzamil is more effective than amiloride in blocking high amiloride-affinity epithelial Na^+ channels, whereas dimethylamiloride is more effective than amiloride in blocking Na^+/H^+ antiport exchange (Kleyman and Cragoe, '88). These results were consistent with the pharmacologic profile characterizing low amiloride-affinity Na^+ channels (Oh and Benos,'92). However, an alternative interpretation was possible. The human NPE cells also display Na^+/H^+ antiport activity (Civan et al., '96). Thus, the observed inhibitions of the RVI could have reflected the actions of amiloride and its analogues on two different transport targets: benzamil could have been blocking high amiloride-affinity Na^+ channels [to which the αβγ-ENaC heterotrimeric channel (Canessa et al., '94) belongs] and dimethylamiloride could have been acting solely on the Na^+/H^+ antiport. Thus, the data indicated the operation of a Na^+ channel, but did not resolve whether the channel conformed to a high- or low-affinity class Na^+ channels, so that its molecular identity was unknown.

TABLE 1. Intracellular composition of intact rabbit ciliary epithelium[1]

	Concentration (mmol/kg intracellular water)		
Component	PE cells	NPE cells	All cells
Na	13 ± 2	16 ± 2	15 ± 3
K	164 ± 12	162 ± 8	162 ± 14
Cl	44 ± 3	48 ± 3	46 ± 5

[1]The entries are the means ± SE taken under baseline conditions from the electron probe X-ray microanalyses of Bowler et al. ('96).

IDENTIFICATION AND REGULATION OF AN EPITHELIAL NA^+ CHANNEL

In the present manuscript, we report finding the expression of an amiloride-sensitive Na^+ channel in the human ciliary body. Total RNA (20 μg) was

extracted (Chomczynski and Sacchi, '87) from the ciliary body of a human eye and separated in a 1%-agarose gel, blotted and hybridized in high-stringency conditions (Coca-Prados et al., '96) with a probe for the expression of transcripts of the αβγ-ENaC heterotrimeric, amiloride-sensitive channel (Canessa et al., '94). The probe chosen was a 1.9-kb DNA probe corresponding to an EcoRI/BamHI fragment of the α-subunit of the rat amiloride-sensitive epithelial sodium channel (a gift of Dr. Cecilia M. Canessa, Yale University).

Figure 1 presents a resulting Northern blot analysis. At the left are the migration positions of standard RNA molecular markers. The arrow at the right indicates the position of the major 3.7-kb transcript specific for the α-subunit mRNA. It is likely that this probe of the αβγ-ENaC epithelial Na^+ channel has detected mRNA from the epithelial cells. However, it should be noted that other cells of the human ciliary body (such as smooth muscle) may also have contributed to the transcript detected.

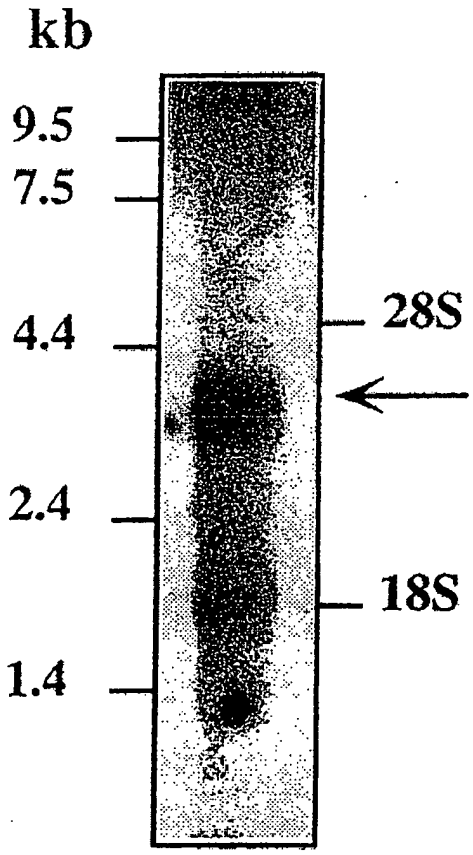

Fig. 1. Expression of an amiloride-sensitive Na^+ channel in the human ciliary body. At left are the migration positions of standard RNA molecular markers. The arrow at the right indicates the position of the major 3.7-kb transcript specific for the α-subunit mRNA.

The results of Figure 1 support the concept that the αβγ-ENaC epithelial Na^+ channel supports reabsorption of Na^+ from the aqueous humor back into the NPE cells. However, it was unclear whether this Na^+-channel activity was expressed under baseline conditions or only after stimulation (in this case, by shrinkage). Recently, we have measured the effects of 1 μM benzamil on parallel aliquots of isotonic suspensions of human NPE cells before and after exposure to 50%-hypotonic solutions, following the approach of Civan et al. ('96). Benzamil had no detectable effect on the baseline isotonic volume at the same concentration which inhibited the RVI (Fig. 2). We conclude that shrinkage activates epithelial Na^+ channels of human NPE cells, as has been noted for other epithelial cells (Okada and Hazama, '88; Wehner et al., '95).

COMPONENTS UNDERLYING VECTORIAL TRANSPORT AT THE NPE-AQUEOUS HUMOR INTERFACE

The foregoing results indicate shrinkage-activated αβγ-ENaC epithelial Na^+ channels participate in reabsorption of aqueous humor back into the NPE cells. Volumetric measurements of immortalized human (ODM) NPE cells indicate that three other mechanisms can also underlie unidirectional reabsorption: a Na^+/H^+ antiport in parallel with a Cl^-/HCO_3^- antiport, a diazide-sensitive Na^+-Cl^- symport, and the bumetanide-sensitive Na^+-K^+-$2Cl^-$ symport (Civan et al., '96).

As noted above, unidirectional secretion at the basolateral surface of the NPE cells is likely to proceed through the Na^+/K^+ exchange pump and through parallel K^+ and Cl^- channels. Different isozymes of the pump appear to be operative in the pars plana and pars pliccata areas of the ciliary epithelium (Ghosh et al., '90). The precise molecular identities of the K^+ channels have not yet been identified, but inward rectifiers, delayed rectifiers and calcium-activated outward rectifiers have been observed with NPE cells (Jacob and Civan, '96). The identity of the Cl^- channels has not yet been unequivocally established. On the basis of their results, Coca-Prados et al. ('96) have suggested that the volume-sensitive protein pI_{Cln} may be regulating a conduit formed by the protein kinase C (PKC)-inhibitable CIC-3 Cl^- channel in human NPE cells. Results obtained with an antibody of P-glycoprotein suggest that this

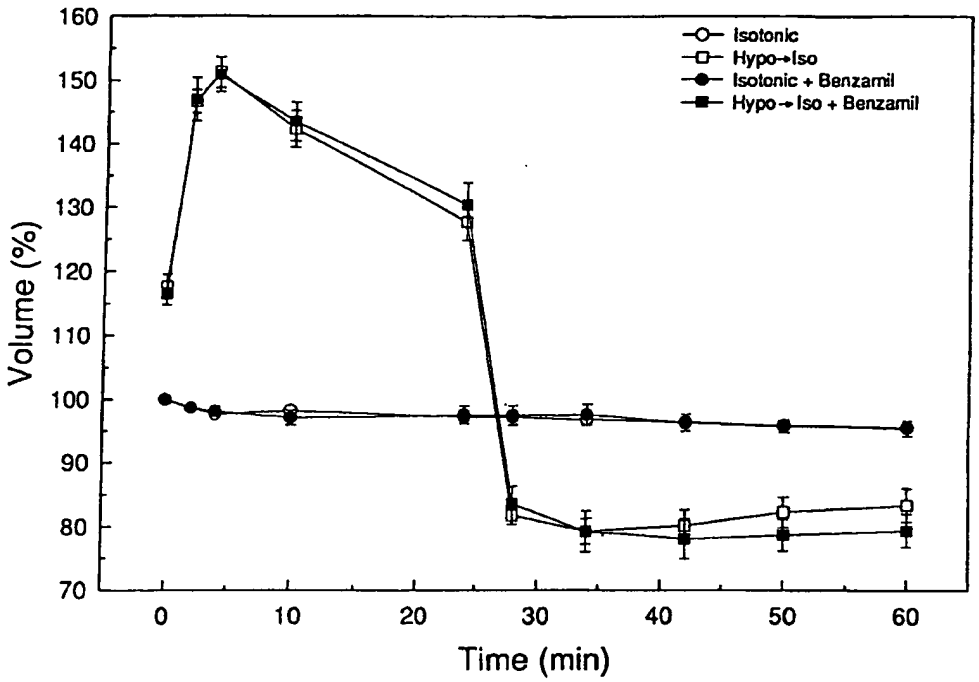

Fig. 2. Effects of benzamil on the regulatory volume increase (RVI) and cell volume in isosmotic suspensions. The cell volumes were measured by electronic cell sorting (Civan et al., '96) in the absence (open symbols) or presence (closed symbols) of 1 μM benzamil. The cell volumes have been normalized to the initial value in isotonic medium. The square symbols present data points (±1 SE) for human NPE (ODM) cells suspended in hypotonic Ringer's solution containing (in mM): 55.0 NaCl, 7.5 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.2 $CaCl_2$, 0.6 $MgCl_2$, 2.4 KCl, 0.6 KH_2PO_4, 15 $NaHCO_3$, and 10.0 glucose. In the absence of added sucrose, the external osmolality was hypotonic (150–160 mOsm), producing swelling and triggering a secondary regulatory reduction in volume over the 4–24 min following suspension. Sufficient sucrose was added 25 min after suspension to bring the osmolality to isotonicity (300–315 mOsm), thereby shrinking the cells until the appearance of a regulatory volume increase (RVI) over the period t = 34–60 min. Measured by linear least-square regression, the rate of swelling during the RVI displayed by the control aliquots was 0.162 ± 0.024%/min (n = 8). Benzamil reduced the rate of the RVI in paired aliquots to 0.012 ± 0.036%/min ($P < 0.025$ by Student's paired *t*-test). In contrast, the same concentration of benzamil had no detectable effect on the cell volume of aliquots suspended in isotonic solution (n = 4). The four isotonic experiments were conducted in parallel with four of the eight RVI experiments and have not been previously reported. The additional four sets of measurements included with the RVI experiments were obtained from the results of Civan et al. ('96).

protein product of MDR1 may also regulate Cl^- currents in bovine NPE cells (Wu et al., '96).

CONJUGATE REGULATION OF UNIDIRECTIONAL SECRETION AND REABSORPTION

With rare exception (Sears, '84), studies of net ciliary epithelial secretion have largely focused on the unidirectional transfer of solutes and water from the stroma to the aqueous humor. However, in principle, we might expect that secretagogues could well have complementary effects on unidirectional secretion and reabsorption. Otherwise, stimulation or inhibition of both antiparallel flows might have no effect on net secretion. As noted above, shrinkage stimulates Na^+-channel activity of human NPE cells. Since ciliary epithelial cells are unlikely to undergo great changes in volume in vivo, we have also examined the potential regulatory roles of other modifiers of net secretion. Inhibition of PKC with staurosporine activates Cl^- channels of human NPE cells (Civan et al., '94; Coca-Prados et al., '95, '96); and activation of PKC has the opposite effect (Civan et al., '94). In order to examine the effects of staurosporine on the reverse flow, we have again turned to the simplest possible experimental model of aqueous humor reabsorption, the regulatory volume increase (RVI) of NPE cells. In contrast to its stimulation of Cl^- secretion, staurosporine inhibits RVI (Civan et al., '96). These conjugate effects of inhibiting PKC activity are illustrated in the cartoon of Figure 3.

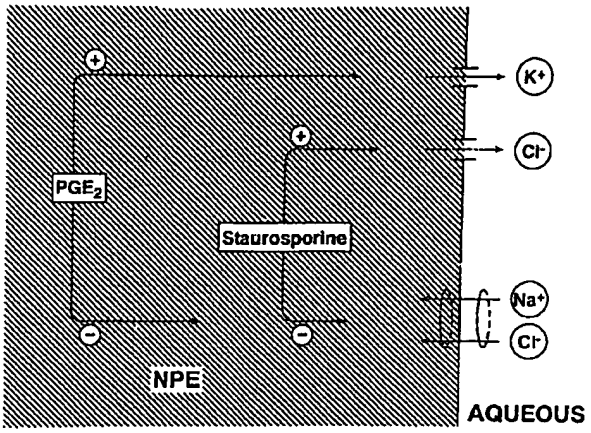

Fig. 3. Postulated regulation of net aqueous humor formation by conjugate modulation of unidirectional secretion and unidirectional reabsorption by the NPE ciliary epithelial cells.

The actions of the arachidonic acid metabolite PGE_2 provide a second, slightly more complex example of the conjugate modification of unidirectional secretion and reabsorption (Fig. 3). PGE_2 also stimulates Cl^- secretion, but its action is indirect, at least in human ODM NPE cells. The prostanoid increases K^+-channel activity (Civan et al., '94), hyperpolarizing the membrane, and thereby providing an increased electrical driving force for Cl^- release into the aqueous humor. Like staurosporine, PGE_2 inhibits the RVI of the NPE cells (Civan et al., '96).

To the extent that the RVI can be taken as an index of reabsorption of aqueous humor by the NPE cells, these results indicate that second messenger cascades can exert complementary actions on unidirectional secretion and reabsorption. Reductions in unidirectional reabsorption as well as increases in unidirectional secretion can enhance the net rate of aqueous humor formation. The converse is also possible, so that future approaches to the medical treatment of glaucoma could well focus on increasing the rate of unidirectional reabsorption in order to reduce net aqueous flow.

ACKNOWLEDGMENTS

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Inhibitors of NHE-1 Na^+/H^+ Exchange Reduce Mouse Intraocular Pressure

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PURPOSE. To test whether blocking the Na^+/H^+ antiport reduces intraocular pressure (IOP) in the mouse.

METHODS. The electrophysiologic approach (the servo-null micropipette system, SNMS) that had been adapted for continuously monitoring IOP in the mouse was used in a study of the effects of a series of transport inhibitors.

RESULTS. Topical application of three direct blockers of Na^+/H^+ exchangers produced comparable reductions in mouse IOP: dimethylamiloride (DMA, -5.0 ± 0.7 mm Hg), ethylisopropylamiloride (EIPA, -4.1 ± 1.0), and BIIB723 (-4.9 ± 1.7 mm Hg). These effects were mediated locally, not systemically, because adding DMA to one eye had no effect on IOP in the contralateral eye. In contrast to the actions of selective inhibitors of Na^+/H^+ exchange, neither the low-potency inhibitor amiloride nor the inhibitor of Na^+-K^+-$2Cl^-$ cotransport bumetanide by itself was effective. Dorzolamide, which slows delivery of H^+ and HCO_3^- to Na^+/H^+ and Cl^-/HCO_3^- antiports, also reduced IOP by 2.9 ± 0.6 mm Hg. After first blocking Na^+/H^+ exchange with DMA, EIPA, BIIB723, or dorzolamide, application of bumetanide produced an additional reduction in IOP of 3.8 to 4.0 mm Hg.

CONCLUSIONS. The first step in formation of aqueous humor is uptake of NaCl by the ciliary epithelial cells from the stroma, possibly by both paired Na^+/H^+ and Cl^-/HCO_3^- antiports and a bumetanide-sensitive Na^+-K^+-$2Cl^-$ symport. The present data are consistent with electron probe x-ray microanalyses of rabbit ciliary epithelium indicating that the antiports are the dominant mechanism. That bumetanide can produce a previously unobserved lowering of IOP when the Na^+/H^+ antiport is also inhibited substantiates a dominant antiport mechanism. (*Invest Ophthalmol Vis Sci.* 2002;43:1897–1902)

Intraocular pressure (IOP) reflects a balance between inflow across the ciliary epithelium and outflow, which largely exits through the trabecular meshwork and Schlemm canal of the primate eye. Inflow is generally considered to proceed in three steps across the bilayered ciliary epithelium[1–9] (Fig. 1): uptake of solute and water by the pigmented ciliary epithelial (PE) cells at the stromal surface, passage through gap junctions to the nonpigmented ciliary epithelial cell (NPE) layer, and transfer from the NPE cells into the aqueous humor of the anterior chamber. At the stromal surface, paired Na^+/H^+ and Cl^-/HCO_3^- antiports[4,6,10,11] and/or a bumetanide-sensitive Na^+-K^+-$2Cl^-$ symport[3,8,12–14] can underlie PE-cell uptake of NaCl, the principal solute of the aqueous humor. Which set of mechanisms dominates the first step in secretion has been unclear.

The mouse has been proposed as a potentially useful animal for studying aqueous humor dynamics, because its outflow tract is structurally closer to that of the human[15] than is that of other commonly used nonprimate species, such as the cow or rabbit. We recently adapted an electrophysiologic technique, the servo-null micropipette system (SNMS), for monitoring IOP in the small mouse eye.[16] With the SNMS, we found that IOP responses in the mouse eye parallel those in the human eye, not only to drugs that alter aqueous humor outflow, but also to those that alter aqueous humor inflow.[16] Furthermore, we have found that SNMS measurements of mouse IOP are sufficiently reliable to permit identification of novel receptor mechanisms that regulate IOP.[17] In the current study, we examined IOP responses in the mouse eye to inhibitors of both sets of transport processes implicated in the initial step of formation of aqueous humor.

MATERIALS AND METHODS

Animals

Black Swiss outbred mice of mixed sex, 7 to 9 weeks old and approximately 30 g in weight, were obtained from Taconic, Inc. (Germantown, NY). Animals were housed in accordance with National Institutes of Health recommendations, maintained under a 12-hour light-dark illumination cycle, and allowed unrestricted access to food and water. IOP measurements were performed at the same time of day (2–6 PM) to minimize diurnal effects on IOP. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

Before all IOP measurements, mice received general anesthesia in the form of intraperitoneal ketamine (250 mg/kg), supplemented by topical proparacaine HCl (0.5%; Allergan, Hormigueros, Puerto Rico).[16]

Servo-Null Micropipette System

The SNMS is an electrophysiologic, nonmanometric method of measuring pressure that we have previously adapted and validated for measuring IOP in the mouse.[16] The exploring, 5-μm micropipette is filled with 3 M KCl solution to ensure that the resistance of the fluid within the tip is much lower than that of the extracellular fluid. The resistance to electrical flow through the micropipette is continuously monitored and is dominated by the electrical resistance at the tip. After entry of the tip into the anterior chamber, the step change in hydrostatic pressure forces aqueous humor into the micropipette, displacing the low-resistance 3-M KCl filling solution from the tip back toward the shank. The resultant increase in electrical resistance generates a signal to a vacuum-pressure pump that produces an equal counterpressure that maintains the position of the aqueous humor-KCl interface at the tip of the micropipette and thus sustains the original electrical resistance. This counterpressure equals the hydrostatic pressure outside

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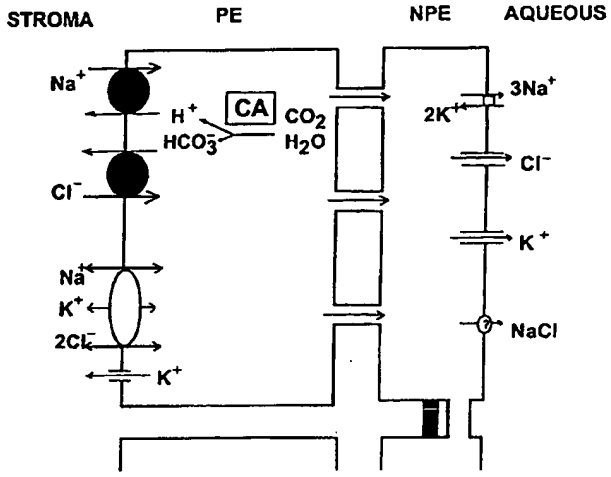

FIGURE 1. Consensus model of aqueous humor formation (modified from Counillon et al.[22] and McLaughlin et al.[26]). Carbonic anhydrase-limited delivery of H^+ and HCO_3^- limits uptake of stromal NaCl through paired antiports. In parallel, NaCl can also enter (or exit[26]) PE cells through the Na^+-K^+-$2Cl^-$ symport. At the contralateral surface, Na^+ and Cl^- can be released from the NPE cells into the aqueous humor through Na^+,K^+-activated ATPase and Cl^- channels, respectively. An electroneutral transporter may also support release into the aqueous humor.

the micropipette tip, in this instance the IOP. The output signal of the servo-null device (Servo-Null Micropressure System model 900A; World Precision Instruments [WPI], Sarasota, FL) was converted to digital form (Duo 18-Data Recording System; WPI), continuously displayed on a monitor, and saved in a computer file at three to five readings per second. Before every measurement, the system was calibrated externally against a mercury manometer in the range from 0 to 50 mm Hg at 5- to 10-mm Hg intervals.

Micropipette Design

Micropipettes were fabricated from borosilicate glass (1.5 mm outer diameter, 0.84 mm inner diameter, WPI) with a puller (Sutter Instruments, San Rafael, CA). The tips were beveled to an outer diameter of 5 μm and a 45° angle with a micropipette beveler (Sutter). When filled with 3 M KCl solution, these micropipettes displayed resistances of 0.25–0.60 MΩ.

Procedure for Measuring IOP

After reaching a stable plane of anesthesia confirmed by absent response to foot pinch, the mice were secured in a surgical stereotaxic device (David Kopf Instruments, Tujunga, CA), with the head positioned to avoid any pressure on the animal that could affect IOP. A heating pad at 37°C (Delta Phase Isothermal Pad, Braintree Scientific, Braintree, MA) maintained body temperature. Topical proparacaine supplemented general anesthesia, and corneal dehydration was prevented by topical normal saline (309 mOsm), as necessary. The ground electrode was placed on the conjunctiva of the same or the contralateral eye, carefully avoiding any pressure on the eye.

The micropipette tip was next placed in the drop of proparacaine on the cornea overlying the pupil, and the output reading from the SNMS was adjusted to zero. The micropipette was then advanced across the cornea (at 20–30° to the optical axis) into the anterior chamber by a cell-penetration positioning system (model LSS 21200; Burleigh Instruments, Inc., Fishers, NY) and a piezoelectric step driver (model PZ100; Burleigh). IOP was monitored after positioning the micropipette tip in the aqueous humor.

The baseline IOP in the present study was 14.2 ± 0.4 mm Hg (n = 113). In measuring drug-induced changes in IOP, each animal served as its own series control. All pressures after drug application were compared with those just before the drug was added.

Statistics

To determine an individual IOP reading, the mean ± SEM was calculated during a 3- to 5-minute recording period. Numbers of experiments or eyes are indicated by the symbol n. The statistical significance of changes in IOP was tested with Student's paired t-test.

Drugs

Drugs were applied topically in 10-μL droplets with a pipette (Eppendorf; Brinkman Instruments, Westbury, NY) at the stated concentrations; total doses are also provided in parentheses. Agents were initially dissolved in dimethyl sulfoxide (DMSO). Unless otherwise stated, the final droplet solution was an isosmotic saline solution (310 mOsm) containing 1% to 8% DMSO and 0.003% benzalkonium chloride (Sigma Chemical Co., St. Louis, MO), commonly used to enhance ocular drug penetration. We have found that the DMSO-benzalkonium solution itself has no effect on mouse IOP at DMSO concentrations as high as 10% (Table 1). DMSO concentrations as high as 15%[18] to 20%[19] have been reported not to alter IOP in rabbits.

We have already reported evidence that changes in mouse IOP produced by our method of topical administration are mediated by local ocular, and not systemic, actions, because unilateral topical application does not alter either pupillary size (1% pilocarpine,[16] 1% tropicamide[17]) or IOP (100 μM adenosine[17]) in the contralateral eyes. Consistent with our earlier observations, we now report that topical application of 1 mM dimethylamiloride (DMA) did not affect the IOP of the contralateral eye (ΔIOP = 0.08 ± 0.40 mm Hg, n = 6, $P \geq 0.8$), but reduced IOP of the treated eye by 3.8 ± 0.5 mm Hg (n = 23, $P <$ 0.001, Table 1).

Among the drugs administered were the selective Na^+/H^+ antiport inhibitors DMA and EIPA (Sigma Chemical Co.). A third such inhibitor used was BIIB723 (Boehringer/Ingelheim, Biberach an der Riss, Germany), which is a member of the BIIB family of Na^+/H^+ antiport blockers.[20] Similar to nearly all other NHE-1 inhibitors, BIIB723 is an acylguanidine, displaying a selectivity for NHE-1 over NHE-2 of approximately 40-fold and an IC_{50} of approximately 30 nM in cardiomyocytes and approximately 100 nM in hamster fibroblasts (Seidler R, unpublished data, 1998–1999). The parent compound (amiloride; Merck, Rahway, NJ) of the amiloride analogues DMA and EIPA is a low-potency inhibitor of both Na^+/H^+ and Na^+/Ca^{2+} antiports and a higher-potency blocker of ENaC Na^+ channels.[21] Bumetanide (Hoffmann-La Roche, Nutley, NJ) is a selective inhibitor of Na^+-K^+-$2Cl^-$ cotransport. Dorzolamide (Trusopt; Merck) is a topical carbonic anhydrase inhibitor.

RESULTS

Single Drug Effects on Mouse IOP

The NHE-1 member of the family of six Na^+/H^+ exchanger (NHE) transporters is known to be the major basis for antiport activity at the basolateral surface of the PE cells facing the stromal surface.[11] DMA, an amiloride analogue with a highly selective inhibitory effect on the NHE-1 antiport,[22] produced a concentration-dependent lowering of IOP (Fig. 2, Table 1). The precise values are uncertain for the threshold droplet concentrations of the drugs used, but DMA was clearly effective at a droplet concentration of 1 mM (2.94 μg, n = 23, Table 1), and a greater lowering of IOP (by 5.0 ± 0.7 mm Hg) was obtained with a droplet concentration of 3 mM (8.82 μg, n = 4; Table 1). Another amiloride analogue, EIPA, displayed the same minimally effective droplet concentration and enhanced lowering of IOP at 3 mM (300 ng; by 4.1 ± 1.0 mm Hg, Table 1). A third acylguanidine antiport inhibitor, BIIB723, produced a maximal hypotensive effect at 3 mM (16.0 μg) of 4.9 ± 1.7 mm Hg,

TABLE 1. Single-Drug Effects of DMA, EIPA, Bumetanide, BIIB723, and Dorzolamide on IOP

Drug	Class	n	Conc.	Dose	ΔIOP (mm Hg)	P
DMA	Na/H antiport inhibitor	3	100 μM	294 ng	+0.9 ± 0.9	
		23	1 mM	2.94 μg	−3.8 ± 0.5	<0.001
		4	3 mM	8.82 μg	−5.0 ± 0.7	<0.01
EIPA	Na/H antiport inhibitor	3	100 μM	300 ng	+0.8 ± 0.2	
		10	1 mM	3.00 μg	−2.6 ± 0.5	<0.001
		6	3 mM	9.00 μg	−4.1 ± 1.0	<0.01
BIIB	Na/H antiport inhibitor	3	10 μM	53.4 ng	−0.4 ± 1.9	
		4	100 μM	534 ng	−2.7 ± 0.4	<0.01
		17	1 mM	5.34 μg	−4.5 ± 0.5	<0.001
		4	3 mM	16.0 μg	−4.9 ± 1.7	
Dorzolamide	CA topical inhibitor	11	55.4 mM	200 μg	−2.9 ± 0.6	<0.001
Bumetanide	Na-K-2Cl symporter blocker	4	10 μM	36.4 ng	−0.2 ± 1.6	
		3	100 μM	364 ng	−0.8 ± 0.7	
		7	1 mM	3.64 μg	−0.7 ± 1.6	
		12	10 mM	36.4 μg	−1.2 ± 0.6	
Contralateral Drugs						
DMA		6	1 mM	2.94 μg	+0.1 ± 0.4	
Vehicle						
DMSO (10%)		5	10%	10.0 μg	−0.3 ± 0.6	

Conc., concentration.

similar to that of DMA (n = 4, Table 1), but displayed a lower minimally effective droplet concentration (100 μM [554 ng]), n = 4, Table 1). The similarity of the effects of BIIB723 at 1 mM (5.34 μg; −4.5 ± 0.5 mm Hg) and 3 mM (16.0 μg; −4.9 ± 1.7 mm Hg) and the similar reductions produced by all three NHE-1 inhibitors at 3 mM suggest that a maximal IOP reduction was achieved of 4.1 to 5.0 mm Hg. We were unable to increase the delivered droplet concentration without substantially increasing the DMSO level, thereby triggering a vehicle-induced change in IOP.

Carbonic anhydrase inhibition reduces the rate of production of H^+ and HCO_3^-, which in turn must slow the rate of delivery of H^+ and HCO_3^- to all cell sites, including the antiports. We have already reported that inhibiting carbonic anhydrase with intraperitoneal acetazolamide lowers mouse IOP (by 11.9 ± 1.3 mm Hg).[16] We have now found that topical application of dorzolamide also reduces IOP, albeit to a lesser extent at the droplet concentration applied (Table 1).

We also tested the effects of amiloride which inhibits NHE-1 antiports at a potency 1 to 2 orders of magnitude lower than the amiloride analogues DMA and EIPA.[11] Consistent with this information, amiloride itself exerted no significant effect on mouse IOP at a droplet concentration of 1 mM (2.30 μg, n = 7, data not shown). To reach a 10-mM concentration, it was necessary to solubilize amiloride in 30% DMSO. After pretreatment with vehicle containing 30% DMSO, subsequent application of 10 mM amiloride in the same concentration of vehicle did not alter that IOP (ΔIOP = −1.0 ± 0.7 mm Hg, n = 4, P > 0.2). Thus, at a concentration 10 times higher than EIPA's minimal effective concentration, amiloride had no effect, consistent with the known ratio of the potency of these inhibitors (3.9:0.07 μM, or ~56) when applied to PE cells.[11]

In contrast to the IOP reductions triggered by the three selective inhibitors of the NHE-1 antiport at droplet concentrations of 0.1 to 3 mM (Table 1), blockage of the Na^+-K^+-$2Cl^-$ symport with droplet concentrations of 0.1 to 10 mM (364 ng to 36.4 μg) bumetanide had no significant effect on IOP (Fig. 2, Table 1).

Sequential Drug Effects on Mouse IOP

Electron microprobe analyses[6] have suggested that inhibition of the Na^+-K^+-$2Cl^-$ symport lowers Cl^- uptake by the ciliary epithelium under conditions in which the turnover rate of the

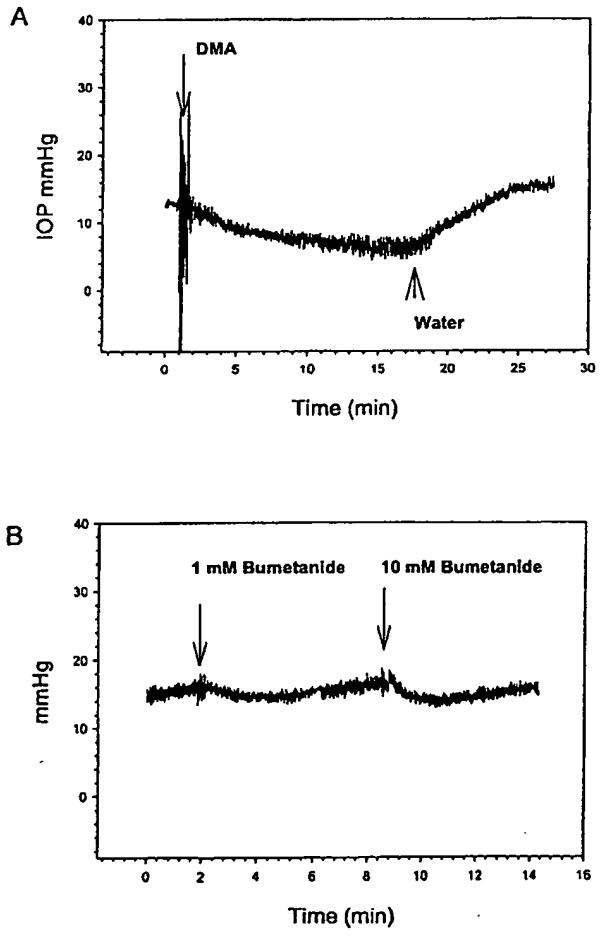

FIGURE 2. Responses of mouse IOP to inhibition of Na^+/H^+ antiports with DMA or to inhibition of Na^+-K^+-$2Cl^-$ antiports with bumetanide. (A) DMA (1 mM, 2.94 μg) lowered IOP. Water was added at the conclusion of this and many other experiments to verify the patency of the micropipette by osmotically raising IOP.[16] (B) Neither 1 mM (3.64 μg) nor 10 mM (36.4 μg) bumetanide by itself significantly altered mouse IOP.

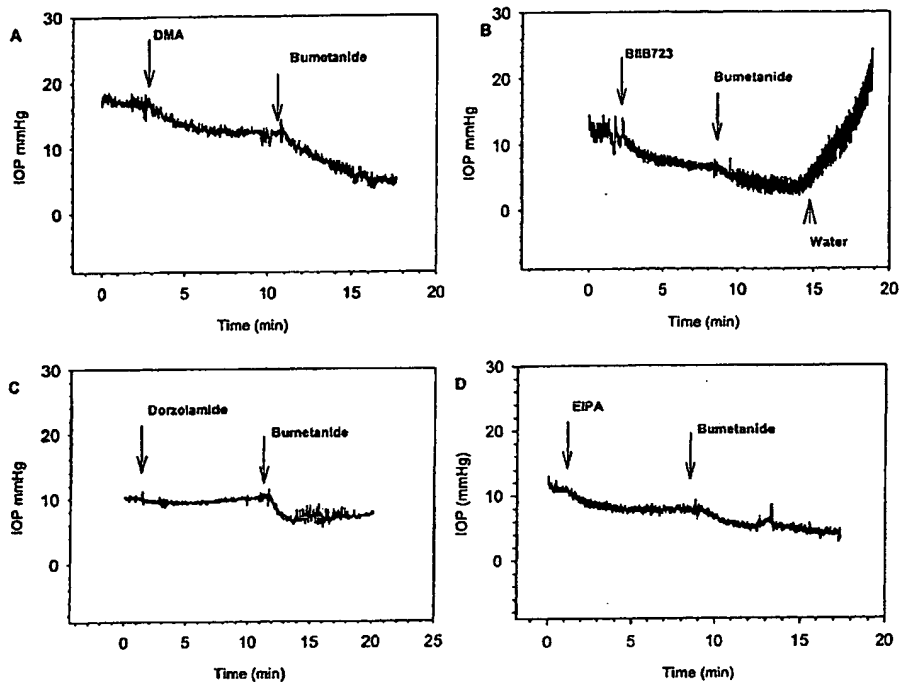

FIGURE 3. Responses to sequential topical addition of direct or indirect inhibitors of Na^+/H^+ antiports, followed by bumetanide: (A) 1 mM (2.94 μg) DMA followed by 1 mM (3.64 μg) bumetanide, (B) 1 mM (5.34 μg) BIIB723 followed by 1 mM (3.64 μg) bumetanide, (C) 55.4 mM (200 μg) dorzolamide followed by 1 mM (3.64 μg) bumetanide, and (D) 1 mM EIPA (3.00 μg) followed by 1 mM (3.64 μg) bumetanide. In each case, bumetanide significantly reduced IOP after prior inhibition of the Na^+/H^+ antiport.

Na^+/H^+ antiport is reduced. To test this hypothesis in vivo, we applied bumetanide after first reducing Na^+/H^+ antiport exchange either directly with acylguanidine inhibitors or indirectly with a carbonic anhydrase inhibitor (Fig. 3, Table 2).

In each case, topical application of the first drug produced the anticipated significant decrease in IOP. Thereafter, the same 10-mM droplet concentration (36.4 μg) of bumetanide, which was ineffective by itself, now triggered significant further lowering of IOP. The entries in Table 2 present the changes in IOP produced first by the initial drug (with respect to baseline) and second by the later addition of bumetanide (in comparison with the previous experimental period). In every case, the secondary application of bumetanide reduced IOP by 3.8 to 4.0 mm Hg (Table 2). Directly inhibiting the Na^+/H^+ antiport with a submaximal 1-mM concentration (5.34 μg) of BIIB723 slightly enhanced the reduction in IOP previously triggered by indirectly inhibiting the antiport with dorzolamide ($\Delta IOP = -0.7 \pm 0.2$ mm Hg, Table 2).

DISCUSSION

The salient findings of the present study are that separate topical application of three different acylguanidine inhibitors of the NHE-1 Na^+/H^+ antiport reduced IOP at 1-mM droplet concentrations, but the far less potent parent compound (amiloride) had no effect on IOP at tenfold higher concentration; application of the selective Na^+-K^+-$2Cl^-$ symport inhibitor bumetanide itself had no significant effect; topical application of the carbonic anhydrase inhibitor dorzolamide reduced IOP in the mouse; and after first inhibiting the NHE antiports either directly with acylguanidine blockers or indirectly with dorzolamide, the subsequent application of bumetanide triggered a highly significant further reduction in IOP of 3.8 to 4.0 mm Hg.

As discussed elsewhere,[17] we do not know the drug concentrations in the very small volume of the mouse anterior chamber (2–4 μL[16,23]) after topical application. However, comparisons of minimally effective droplet concentrations of purinergic drugs with their published K_i suggest that the penetrance (defined as the aqueous-to-droplet concentration ratio) is commonly approximately 1:100 to 1:1000.[17] To extrapolate these values for purinergic drugs to the acylguanidine blockers and bumetanide is necessarily speculative. However, as discussed elsewhere,[17] this apparent penetrance of drugs in the mouse eye is not very different from the approximately 1:100

TABLE 2. Effects on IOP of Sequential Medications

First Drug / Second Drug	n	Conc. of First Drug/ Second	ΔIOP (mm Hg) (after baseline)	P	ΔIOP (mm Hg) (after first drug)	P
Dorzolamide (CA inhibitor)/ Bumetanide (symport inhibitor)	4	55.5 mM (200 μg)/ 10 mM (36.4 μg)	−2.0 ± 0.4	<0.05	−3.9 ± 1.0	<0.05
BIIB (Na^+/H^+ antiport inhibitor)/ Bumetanide (symport inhibitor)	6	1 mM (5.34 μg)/ 10 mM (36.4 μg)	−2.9 ± 1.0	<0.05	−3.9 ± 0.9	<0.01
DMA (Na^+/H^+ antiport inhibitor)/ Bumetanide (symport inhibitor)	6	1 mM (2.94 μg)/ 10 mM (36.4 μg)	−4.0 ± 0.8	<0.01	−3.8 ± 0.7	<0.01
EIPA (Na^+/H^+ antiport inhibitor)/ Bumetanide (symport inhibitor)	6	1 mM (3.00 μg)/ 10 mM (36.4 μg)	−2.4 ± 0.6	<0.01	−4.0 ± 0.6	<0.01
Dorzolamide (CA inhibitor)/ BIIB (Na/H antiport inhibitor)	7	55.4 mM (200 μg)/ 1 mM (5.34 μg)	−3.5 ± 0.9	<0.01	−0.7 ± 0.2	<0.01

Conc., concentration.

penetrance of drugs topically applied to rabbits and primates, as well. By this measure, the minimally effective droplet concentration of 1 mM for DMA and EIPA (Table 1) may have corresponded to approximately 1 to 10 μM in the aqueous humor, and the minimally effective droplet concentration of 100 μM for BIIB723 may have corresponded to aqueous humor concentrations of 0.1 to 1 μM. This difference may arise from a higher penetrance for BIIB723, because the IC_{50} observed for this drug (30–100 nM; Seidler R, unpublished results, 1998–1999) is similar to that of EIPA (50 nM[24]). Although BIIB723 may penetrate more effectively than DMA or EIPA, it is likely that all three NHE-1 inhibitors exerted a maximal effect at 3 mM (see first paragraph of Results), uniformly reducing IOP by 4.1 to 5.0 mm Hg.

The first step in aqueous humor formation is electroneutral uptake of NaCl from the stroma of the ciliary processes by the PE cells of the ciliary epithelium and can be mediated by either paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- exchangers[4,6,10,11] or an Na^+-K^+-$2Cl^-$ cotransporter.[3,8,12–14] Consensus has not yet been reached concerning the relative importance of these two transfer mechanisms. However, electron probe x-ray microanalyses of the elemental compositions of rabbit ciliary epithelium in vitro have suggested that the paired antiports can predominate, at least under certain conditions, and that the bumetanide-sensitive symport can support either uptake or release of solute, depending on the ambient thermodynamic driving force.[6] This interpretation is consistent with the observation that inhibition of the Na^+-K^+-$2Cl^-$ symport with bumetanide has no significant effect on inflow or IOP in the cynomolgus monkey.[25] However, the putative role of Na^+/H^+ and Cl^-/HCO_3^- antiports in regulating mammalian IOP has not previously been tested in vivo.

In the present work, we tested three predictions based on the microprobe analyses.[6] First, if the paired antiports are the dominant mechanism in the first step of aqueous humor formation, blocking one or the other antiport should reduce inflow and thereby IOP. This prediction was met by the ocular hypotensive effects of three different acylguanidine NHE-1 inhibitors (Fig. 2, Table 1). Second, if the Na^+-K^+-$2Cl^-$ symport plays a supplemental role in supporting either uptake or release at the stromal surface, blocking the symport would be expected to have little effect on inflow. Consistent with this prediction, we have confirmed in the mouse that bumetanide alone has no significant effect on IOP, in agreement with the earlier observation in cynomolgus monkeys.[25] Third, when the paired activity of the antiports is blocked, the major mechanism supporting NaCl uptake from the stroma should be the Na^+-K^+-$2Cl^-$ symport. Under these conditions, bumetanide is predicted to have a substantial effect on secretion (see Figures 2 and 3 of McLaughlin et al.[6]). Indeed, the same concentration of bumetanide which was by itself ineffective now uniformly reduced mouse IOP, after either direct NHE inhibition with the acylguanidine compounds or after the carbonic anhydrase inhibitor dorzolamide, which probably inhibits NHEs indirectly by reducing delivery of H^+ and HCO_3^- to the antiports. The IOP recordings in the current study, limited to 12 to 20 minutes largely because of the general anesthesia requirement, establish roles for the antiports, but additional research is needed to learn whether antiport inhibition is an effective strategy for long-term IOP control.

IOP reflects both the inflow and outflow of aqueous humor. Because present methodology permits only IOP measurements in the mouse, the current results can neither exclude an outflow effect nor unambiguously prove that the paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- antiports are the dominant mechanisms underlying the first step in formation of aqueous humor. However, the data are consistent with the latter antiport hypothesis and further lead to the proposal that bumetanide can have a previously unobserved role in lowering IOP if coupled to inhibition of the NHE exchangers.

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$(dT)_{8-18}$ and poly(rC) · oligo(dG)$_{8-18}$. Ten microliters of virus (which was concentrated 100-fold by centrifugation and then suspended in 50 mM tris-HCl, pH 7.3, 10 mM dithiothreitol, and bovine serum albumin, 1 mg/ml) were incubated with 10 μl of 50 mM tris-HCl, pH 7.3, 10 mM dithiothreitol, 0.06 percent Triton X-100 for 10 minutes at 2°C. A portion (20 μl) of assay mixture was added, and the resulting 40-μl reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by the addition of 0.3 ml of 80 mM sodium pyrophosphate, 25 μl of bovine serum albumin (5 mg/ml), and 0.5 ml of 25 percent trichloroacetic acid at 2°C. Acid-insoluble material was collected on glass fiber filters, washed extensively with 10 percent trichloroacetic acid, dried, and dissolved in NCS tissue solubilizer; the radioactivity was then counted in toluene-based scintillation fluid. The reaction mixtures with poly(rA) · oligo(dT)$_{9-18}$ and poly(dA) · oligo $(dT)_{10-18}$ consisted of: 50 mM tris-HCl, pH 7.3, 90 mM KCl, 10 mM dithiothreitol, 0.1 mM each of deoxyriboadenosine triphosphate (dATP), deoxyriboguanosine triphosphate (dGTP), deoxyribocytidine triphosphate (dCTP), 2.3 μM ^{3}H-labeled thymidine triphosphate (40,000 counts/min per picomole), 20 μg of poly(rA) or poly(dA) per milliliter, 20 μg of oligo(dT)$_{10-18}$ per milliliter, and either 5 mM $MgCl_2$ or 0.4 mM $MnCl_2$. The reaction mixture containing poly(rC) · oligo(dG)$_{8-18}$ consisted of: 50 mM tris-HCl, pH 7.3, 50 μg of actinomycin D per milliliter, 15 mM KCl, 10 mM dithiothreitol, 10.7 μM ^{3}H-labeled dGTP (6500 counts/min per picomole), 40 μg of poly(rC) per milliliter, 40 μg of oligo(dG)$_{10-18}$ per milliliter, and either 25 mM $MgCl_2$ or 0.4 mM $MnCl_2$.

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Sodium-Specific Membrane Channels of Frog Skin Are Pores: Current Fluctuations Reveal High Turnover

Abstract. *The reversible sodium transport blocker amiloride causes current fluctuations at the apical membrane of the outer stratum granulosum of frog skin. Their power density spectra reveal that single transport sites translocate more than 10^6 sodium ions per second, which indicates a pore mechanism. The density of open plus amiloride-blocked pores is in the order of 10^8 pores per square centimeter of skin area with 60 millimolar sodium and 18 micromolar amiloride in the outer solution.*

Carrier- and pore-mediated transport through biological membranes can be distinguished by the turnover number of individual transport sites (*1*). While a carrier molecule, which has to move through the lipid phase of the membrane, is not likely to transport more than 10^4 ions or molecules per second, the transport rate of a pore can be several orders of magnitude larger (*2*). Applying this idea to the Na-selective membrane of frog skin, we have attempted to determine the Na turnover of individual transport sites by an evaluation of current fluctuations. The fluctuations were introduced artificially by addition of the drug amiloride, a pyrazine diuretic known to block Na transport reversibly from the outside (*3*). A reversible blocker can be expected to randomly interrupt the Na turnover of individual transport sites. A site will then either conduct fully or, when blocked, not conduct at all. The continuous current i passing one site is thus chopped up into small current pulses of varying duration but equal amplitude (i). The pulses add up to a mean current which on close inspection will show random fluctuations. The mean current per square centimeter will be

$$I_{Na} = iMP_o \qquad (1)$$

where M (cm^{-2}) is the mean density of unblocked plus amiloride-blocked transport sites. The steady-state probability P_o represents the fraction of M not blocked by amiloride and MP_o the mean density of open sites. Statistical evaluation of the fluctuations permits computation of i and thus the Na turnover of individual transport sites in the open state.

Isolated abdominal skin of *Rana esculenta* was used at room temperature. It was mounted in a Lucite chamber which left 3 cm^2 exposed to the bathing solutions. The outer solution was K or Na sulfate Ringer solution containing varying concentrations of amiloride. The inner one was K sulfate Ringer solution, which can be expected to depolarize the K-selective inward-facing membranes of the epithelium and to increase their conductance. Thus, transepithelial resistance and potential were largely determined by the apical membrane of the stratum granulosum (*4*). The current component that did not pass the Na-specific channels was determined as the current flowing in the presence of 35 μ*M* amiloride, and was subtracted from the total current to obtain the transcellular Na current, I_{Na}.

Transepithelial voltage was clamped to 0 mv by a voltage clamp circuit with continuous feedback. The input stage of the voltage-sensing amplifier was designed around a matched pair of low-noise transistors (National Semiconductor 2N4250) to minimize feedback current fluctuations arising from this stage. The open-loop clamp gain was 25,000. The short-circuit current was amplified with a gain of 50 μv/na, fed through a high-pass RC-filter with a characteristic frequency of 0.007 hertz, amplified 400 times, and recorded on magnetic tape. Recording periods were 10 to 30 minutes for each amiloride concentration.

The amplified a-c current signal was sampled from the magnetic tape at frequencies of 50 and 5000 hertz by use of an anti-aliasing filter of the Butterworth type (72 db per octave, characteristic frequency set at 80 percent of the maximal analyzed frequency). The digitized signal was divided into 20 records of 4096 words each. A power density spectrum was computed from each record on an IBM 370/58 by use of a fast Fourier transform program. The spectra of 20 records were averaged.

Figure 1A shows five power density spectra in the range 0.3 to 100 hertz. Curves a to d were obtained with a sodium activity of $(Na)_o$ = 60 m*M* in the outer solution and amiloride concentrations of $(A)_o$ = 1.4, 4.3, 12.7, and 35 μ*M*. Spectrum e was obtained with $(Na)_o$ = 0. Spectra a to d are of the Lorentz type expected for exponential relaxation phenomena. They obey the relationship

$$S = S_o/[1 + (f/f_c)^\alpha] \qquad (2)$$

where S is the power density (amp^2 sec), S_o the plateau value, f the frequency (hertz), and f_c the corner frequency. The exponent α was found to be in the range 1.75 to 2 (1.8 in Fig. 1A). It is evident

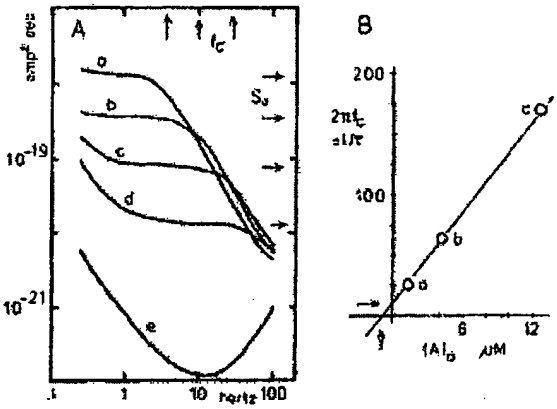

Fig. 1. (A) Power density spectra obtained from 3 cm² of epithelium. Only the parts of the recorded spectra that are relevant for the arguments of this report are shown. (Curves a to d) Increasing amiloride concentrations of $(A)_o$ = 1.4, 4.3, 12.7, and 35 μM at 60 mM $(Na)_o$. (Curve e) Sodium-free outer solution (Na replaced by K). The corresponding values of transcellular Na current are (from curve a to curve e) 41, 24, 9, 0, and 0 μa per 3 cm². The transcellular Na current at $(A)_o$ = 0 was 78 μa per 3 cm² (spectrum not shown). Plateau values (S_a) and corner frequencies (f_c) are indicated with horizontal and vertical arrows; f_c was read off where the power density equaled half of the plateau value. The f_c of curve d (for which I_{Na} was zero) was not evaluated because the α value of this curve fell clearly below the expected range. (B) Relationship between corner frequency and amiloride concentration (see Eq. 4a). Arrows point to the intercepts where k_2 and K_A' are read off. The linear dependence of $1/\tau$ on $(A)_o$ shows, in retrospect, that neglecting the term $k_1(R)$ in Eq. 4 was justified.

that the plateau value, S_a, of the power spectrum decreases with increasing amiloride concentration while the corner frequency f_c increases.

Following a derivation in Verveen and DeFelice (5), we may set $2\pi f_c$ equal to the chemical rate $(1/\tau)$ of the current-modulating process. In the simplest possible case this process is due to the random formation and disintegration of a transport-blocking complex between amiloride (A) and the transport site (R)

$$A + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AR$$

where AR is the blocked site; k_1 and k_2 are rate constants, and their ratio $K_A = k_2/k_1$ is the dissociation constant of AR. With $M = R + AR$, where R and AR are the densities of unblocked and blocked sites, we find from the law of mass action the steady-state probabilities that a site is unblocked or blocked

$$P_0 = R/M = 1/[1 + (A)_m/K_A]$$
$$P_A = AR/M = P_0(A)_m/K_A \quad (3)$$

where $(A)_m$ is the effective amiloride concentration at the outer surface of the membrane. This blocking mechanism implies the following relationship between chemical rate and equilibrium concentrations of A and R (6)

$$1/\tau = k_1(A)_m + k_2(R) + k_2 \quad (4)$$

in which (R) is the equilibrium concentration of unblocked transport sites, expressed in the same units as $(A)_m$. In cases where (R), which decreases with increasing $(A)_m$, is numerically negligible compared to $(A)_m$, a linear relationship between amiloride concentration and chemical rate will be expected. We thus obtain the prediction

$$2\pi f_c = 1/\tau = k_1(A)_m + k_2 =$$
$$k_1\beta(A)_o + k_2 \quad (4a)$$

where $\beta = (A)_m/(A)_o$ is the unknown partition coefficient of amiloride between the outer bulk solution and the space at the outer surface of the membrane. After substituting β into Eq. 3 and combining Eq. 3 with Eq. 4a, we find the "on" and "off" probabilities to be

$$P_0 = 1/[1 + (A)_o/K_A'] = k_2\tau$$
$$P_A = P_0(A)_o/K_A' = k_1'\tau(A)_o \quad (3a)$$

The linear relationship between f_c and $(A)_o$ predicted by Eq. 4a was found in most experiments (for example, see Fig. 1B). This shows that channel blocking by amiloride can be described in terms of simple, bimolecular kinetics, and that $k_2(R)$ in Eq. 4 is numerically negligible. The rate constant k_2 and the "apparent" constants $k_1' = k_1\beta$ and $K_A' = K_A/\beta$ can be estimated from the plot. We found $1/k_2$, the mean lifetime of the complex AR, to be in the order of 100 msec at room temperature. The apparent dissociation constant K_A' was close to 1 μM at 60 mM $(Na)_o$. It decreased with decreasing $(Na)_o$, as expected if Na and amiloride compete for transport sites (7). The apparent constant k_1' was found to be in the order of 10^7 liter mole^{-1} sec^{-1} at 60 mM $(Na)_o$.

For randomly blocked transport sites with unequal on and off probabilities (5) the plateau value of the power density spectrum (Eq. 2) is, in our notation, given by

$$S_a = 4M^2P_0P_A\tau a \quad (5)$$

in which a is the experimental membrane area. Substituting Eq. 1 into Eq. 5, we obtain

$$S_a = 4I_{Na}P_A\tau i a \quad (5a)$$

Further combination with Eqs. 3a and 4a yields an expression for the amplitude of individual current pulses

$$i = \frac{S_a}{4aI_{Na}} \cdot \frac{k_1\beta}{(A)_o}[(A)_o + K_A']^2 \quad (6)$$

For $(Na)_o$ = 60 mM, values in the range 0.3 to 0.5 pa were computed, which correspond to turnover numbers of 1 to 3 × 10^6 Na ions per second for individual transport sites. These turnover numbers are much larger than the values of 10^4 ion/sec which are expected (1) and were observed (8) for shuttle-type mobile carriers, like valinomycin, which have to diffuse through the lipid phase of the membrane. Turnover rates of nonshuttle-type carriers, where the carrier molecule rotates or where only a part of the carrier molecule moves, have, to our knowledge, not yet been obtained experimentally. However, since such carriers will also have to overcome the viscosity and the electrostatic energy barrier of the lipid phase, low turnover rates will be expected in this case too. We feel justified, therefore, in concluding that in the Na-selective membrane of frog skin, transport occurs through pores, which are the only high-rate translocators presently known (9). This result is at variance with a previous conclusion by Biber and Sanders (10), who maintained that the Na transport is carrier mediated.

The mean density of open plus amiloride-blocked Na pores can be calculated from

$$M = I_{Na}/(iP_a) = 4aI_{Na}^2P_A\tau/(P_aS_a) \quad (7)$$

It was found to be in the range 0.7 to 2 × 10^8 pores per square centimeter of membrane area at 60 mM $(Na)_o$. When $(Na)_o$ was lowered to 15 mM by substitution with K, larger pore densities were computed, but did not exceed 3 × 10^9 pores per square centimeter. Extrapolation to $(Na)_o \approx 0$ shows that the total number of pores (N) will be below 5 × 10^8 cm^{-2}. This density corresponds to less than 50 pores per square micrometer of membrane area if a homogeneous distribution of pores over the apical membranes of all cells of the outer stratum granulosum may be assumed.

If, in the absence of amiloride, all pores were permanently open, a Na current $I_{Na} = iN = 2000$ $\mu a/cm^2$ would result at 60 m*M* $(Na)_o$. This current is 75 times larger than the I_{Na} value of 26 $\mu a/cm^2$ actually observed (see legend to Fig. 1A). We deduce from this observation that even in the absence of amiloride the pores are not permanently open (*11*).

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2. We suggest that (i) the term carrier be applied only to translocators where the transfer-mediating moiety moves through the lipid phase; (ii) the term pore be applied to water-filled pathways that permit diffusional transport, which can, however, involve binding of ions to parts of the pore structure; and (iii) different terms be used for translocators that do not meet these specifications [see also (*9*)].
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9. Theoretically, translocators that combine properties of carriers and pores are conceivable. For instance, at a transport site the membrane may effectively be thinned down to a narrow protein structure, part of which can bind an ion selectively and transfer it by a rotational movement in low-viscosity surroundings ["translocase"; see P. Mitchell, *Nature (London)* **180**, 134 (1957)]. Transfer rates may be high, although this is not very likely in view of the low turnover numbers of most enzymes. H. Passow (personal communication) has estimated turnover numbers of 2×10^6 sec^{-1} for the anion "carrier" of erythrocyte membranes, which might fulfill the structural requirements mentioned above. It is an open question of nomenclature how such translocators are to be classified. They resemble pores because the larger part of the membrane's diameter is passed by diffusion through a hydrophilic channel, and they resemble carriers because movement of a membrane component is essential for the transfer event. In the spirit of (*2*) we would not classify such structures, if they exist, as carriers. They may be viewed as pores in which the selectivity filter [B. Hille, *J. Gen. Physiol.* **58**, 599 (1971)] constitutes a peculiar energy barrier.
10. T. U. L. Biber and M. L. Sanders, *J. Gen. Physiol.* **62**, 529 (1973).
11. A. W. Cuthbert, *J. Physiol. (London)* **228**, 681 (1973)] computed, from amiloride binding data, a channel density of 400 μm^{-2} [lower limit at 2.5 m*M* $(Na)_o$], and from this calculated turnover rates for individual Na channels of 2000 sec^{-1} at 2.5 m*M* $(Na)_o$ and 8000 sec^{-1} at 115 m*M* $(Na)_o$ [upper limits]. These turnovers are integrated over open and closed times of a channel and therefore cannot be directly compared with our results.
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Size Limit of Molecules Permeating the Junctional Membrane Channels

Abstract. *The permeability of the cell-to-cell membrane channels in salivary gland cell junction* (Chironomus thummi) *was probed with fluorescent-labeled amino acids and synthetic or natural peptides. Molecules up to 1200 daltons pass through the channels with velocities depending on molecular size. Molecules of 1900 daltons or greater do not pass. This passage failure seems to reflect the normal size limit for junctional channel permeation; the channels continue to be permeated by the molecules up to 1200 daltons when these are mixed with the nonpermeant molecules. From this size limit a channel diameter of 10 to 14 angstroms is estimated.*

Since the finding that fluorescein, a molecule of 330 daltons, passes through a cell junction of low electrical resistance (*1*), other fluorescent and colorant substances have been used to probe junctional permeability properties (*2*, *3*). However, the range of questions that could be studied has been restricted by the small number of useful probes available. We have now enlarged the repertoire of probes with the aim of determining the size limit of molecules permeating the junctional membrane channels (*4*).

We set out to construct fluorescent conjugates which incorporate some of the desirable features of the popular tracer fluorescein, such as water solubility, nontoxicity, low cytoplasmic binding, and high fluorescent yield. To obtain conjugates of well-defined structure, we sought, for the nonfluorescent backbone, not only a molecule of known structure but one with few reactive sites, preferably only one. Thus, the primary amine group of the synthetic and natural peptides listed in Table 1 was coupled with the fluorescent dyes fluorescein isothiocyanate (FITC), dansyl chloride (DANS), or lissamine rhodamine B (LRB) (*5*). The conjugation reactions were carried out at room temperature in an aqueous-acetone solvent made alkaline with $KHCO_3$. The products were purified by ion-exchange and gel-permeation chromatography. The criterion for purity was the formation of a single fluorescent spot in paper electrophoresis. Amino acid analysis and end-group analysis were performed on all peptides, except microperoxidase. The purified compounds were dissolved in water and the *p*H was adjusted to about 7 with KOH or HCl.

The solutions of the fluorescent probes were injected into cells of isolated *Chironomus* salivary glands (mid-fourth instar) with the aid of a micropipette and a pneumatic pressure system (*6*); the spread of the fluorescence inside the cells (excited with wavelengths of 460 nm for FITC, 340 nm for DANS, and 540 nm for LRB) was observed and photographed in a microscope darkfield, or, for velocity determinations, the spread was viewed and videotaped with the aid of an image intensifier-television system (*7*). The fluorescent emissions of FITC and DANS peak at 520 and 525 nm (yellow-green) and that of LRB at 590 nm (red). Thus, in experiments where two tracers were injected together, the LRB was easily distinguished from either of the other two by the use of appropriate filters. In some cases, the tracer studies were combined with measurement of electrical coupling. Electrical current was then passed between the exterior and the interior of the cell injected with the tracer, and the resulting changes in membrane potential were measured in this cell and the immediate neighbor with the use of three microelectrodes (*1*).

The results obtained with the various tracers are summarized in Table 1. The amino acids and peptides with sizes less than or equal to 1158 daltons passed through the junction. Their fluorescence spread from the site of injection throughout the injected cell and into the cell neighbors at rates inversely related to molecular size. The tracers generally crossed several cell junctions on either side. The arrival of a tracer at the junction was marked by an abrupt change in the velocity of the fluorescence spread. With molecules less than or equal to 380 daltons, passage through the first junction became detectable within a few seconds of the tracer's arrival at the junction; and the fluorescence on the two sides of the junction appeared to equalize within 1 to 10 minutes. With molecules between 593 and 1158 daltons, the transit through junction was slower (tak-

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Effects of Adenosine Agonists on Intraocular Pressure and Aqueous Humor Dynamics in Cynomolgus Monkeys

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The effects of single or multiple topical doses of the relatively selective A_1 adenosine receptor agonists (R)-phenylisopropyladenosine (R-PIA) and N^6-cyclohexyladenosine (CHA) on intraocular pressure (IOP), aqueous humor flow (AHF) and outflow facility were investigated in ocular normotensive cynomolgus monkeys. IOP and AHF were determined, under ketamine anesthesia, by Goldmann applanation tonometry and fluorophotometry, respectively. Total outflow facility was determined by anterior chamber perfusion under pentobarbital anesthesia. A single unilateral topical application of R-PIA (20–250 μg) or CHA (20–500 μg) produced ocular hypertension (maximum rise = 4·9 or 3·5 mmHg) within 30 min, followed by ocular hypotension (maximum fall = 2·1 or 3·6 mmHg) from 2–6 hr. The relatively selective adenosine A_2 antagonist 3,7-dimethyl-1-propargylxanthine (DMPX, 320 μg) inhibited the early hypertension, without influencing the hypotension. Neither 100 μg R-PIA nor 500 μg CHA clearly altered AHF. Total outflow facility was increased by 71% 3 hr after 100 μg R-PIA. In conclusion, the early ocular hypertension produced by topical adenosine agonists in cynomolgus monkeys is associated with the activation of adenosine A_2 receptors, while the subsequent hypotension appears to be mediated by adenosine A_1 receptors and results primarily from increased outflow facility.

Key words: adenosine; agonists; antagonists; aqueous; facility; hypertension; hypotension; monkeys; ocular; outflow; pressure; receptors.

1. Introduction

A single topical dose of the relatively A_1 selective adenosine agonists R-PIA and CHA reportedly affected IOP biphasically in rabbits, with ocular hypertension at 30 min followed by pronounced IOP reduction (Camras et al., 1994; Crosson 1992; Crosson and Gray, 1994a, b; Crosson 1995). The physiological mechanism for the ocular hypertension was not clear, but it was inhibited by the adenosine A_2 antagonist DMPX without influencing the IOP reduction. The ocular hypotension was inhibited by the A_1 antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) but not by the cyclooxygenase inhibitor indomethacin, suggesting that the hypotension was mediated by adenosine A_1 receptors rather than by production of prostaglandins.

The anatomy and physiology of the aqueous humor formation and drainage apparatus in primates and rabbits differ in important respects (Poyer, Gabelt and Kaufman, 1992), the effect of adenosine agonists and antagonists on IOP and aqueous humor dynamics has not been studied in primates, and the hypotensive response could be associated with any one or more of the four parameters that affect IOP (decreased AHF, increased outflow facility, increased uveoscleral outflow and decreased episcleral venous pressure). We report here the effects of single or multiple doses of R-PIA or CHA on aqueous humor dynamics in normotensive cynomolgus monkey eyes in vivo.

2. Materials and Methods

Animals

Cynomolgus monkeys (*Macaca fascicularis*), weighing 1·6–5·6 kg, were studied. All investigations were in accordance with University of Wisconsin and NIH guidelines, and with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

Anesthesia for IOP and AHF measurements was induced with i.m. ketamine (10 mg kg^{-1}) and maintained with supplemental i.m. injections as required (usually 5 mg kg^{-1} every 30–45 min). Anesthesia for total outflow facility was induced by i.m. ketamine (10 mg kg^{-1}) followed by i.m. pentobarbital-Na (35 mg kg^{-1}).

Drug Preparation and Administration

R-PIA, CHA and DMPX were obtained from Research Biochemical Inc., Natick, MA, U.S.A., and dimethyl sulfoxide (DMSO) from Research Industries Corporation, Salt Lake City, UT, U.S.A. R-PIA, CHA and DMPX solutions were prepared in DMSO and

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water as follows: the 20 μg and 100 μg doses of R-PIA or CHA were dissolved in 10 μl of 15% DMSO; 250 μg of R-PIA was dissolved in 25 μl of 50% DMSO; 500 μg of CHA was dissolved in 20 μl of 25% DMSO; 160 μg of DMPX was dissolved in 32 μl of 20% DMSO. Vehicle was the corresponding concentration of DMSO used to prepare the drug solutions. R-PIA, CHA or their vehicles were administered in 5 μl drops. DMPX or vehicle was administered in 8 μl drops.

For IOP and AHF studies, drugs were administered topically to the central cornea of the supine monkey at 30 sec intervals, with blinking prevented between each drop. For single dose experiments or on days 1 and 4 in multiple dose experiments, drugs or vehicles were administered under ketamine anesthesia. On days 2 and 3 in multiple dose experiments, the drugs or vehicles were administered twice daily (at 09:00 and 15:00) to fully conscious manually restrained monkeys.

Intraocular Pressure Measurement

IOP was determined with a 'minified' Goldmann applanation tonometer (Kaufman and Davis, 1980), using 'Half and Half®' creamer solution (Borden Inc. Columbus, OH, U.S.A.) as the tear film indicator, with the monkey lying prone in a head holder and the eyes positioned at 4 to 8 cm above the heart. For each eye, three IOP readings taken 5–10 min apart were averaged as a baseline before administration of drugs and vehicles. Single IOP readings were taken at 0·5, 1, 2, 3, 4, 5 and 6 hr after 20–250 μg R-PIA or 20–500 μg CHA for the single dose protocol; at 0·5, 2, 4, 6 and 9 hr on day 1 and 0·5, 1, 2, 4, 6 and 9 hr on day 4 for the multiple treatment protocol; at 0·5, 1, 2, 3, 4, 5, and 6 hr after 100 μg R-PIA or 500 μg CHA for the bilateral agonist and unilateral antagonist protocol (160 μg DMPX was administered 30 and 60 min before agonist); or at 1, 2, 3, 4, 5, 6, and 7 hr after the first dose of DMPX for the antagonist-only protocol. At least one week elapsed between IOP experiments in a given animal.

Aqueous Humor Flow Measurement

At 1700 hr, background fluorophotometric readings were taken. Immediately thereafter, one drop of 0·5% proparacaine hydrochloride (Alcaine®, Alcon Laboratories, Ft. Worth, TX, U.S.A.) was administered to the central cornea of both eyes of each supine monkey (to enhance corneal penetration of fluorescein), followed 5 min later by four 2 μl drops of 2% fluorescein-Na (Alcon) at 30 sec intervals. Drugs or vehicles were administered at 0900–1000 hr the next day. Corneal and anterior chamber fluorescence were measured using a Coherent Fluorotron Master® scanning ocular fluorophotometer. The average of two scans was taken for each eye every 30 minutes for 6 hr after agonist administration for all protocols. In the agonist-only protocol, no AHF baseline was measured. During the R-PIA experiment, AHF in three monkeys was measured for only 3 hr due to subsequent equipment problems. In the bilateral agonist and unilateral antagonist protocol, baseline AHF was determined 1 week before and 7 weeks after bilateral 500 μg CHA and unilateral 320 μg DMPX administration (160 μg DMPX was administered 30 and 60 min before 500 μg CHA), with no experiments performed before the first baseline or between drug administration and the second baseline. No significant ipsilateral or contralateral differences were observed within or between the two baselines, so the average of the two baselines within the corresponding time periods was used. In all other protocols, at least 4 weeks elapsed between AHF experiments in a given animal. AHF was calculated from the scans by a modified method of Jones and Maurice (Jones and Maurice, 1966; Gabelt et al., 1995).

Outflow Facility Measurement

Total outflow facility was determined by 2-level constant pressure perfusion of the anterior chamber with Bárány's mock aqueous humor (Bárány, 1963), using a one-needle technique and correcting for internal apparatus resistance (Bárány, 1965). Most monkeys had undergone more than one prior anterior chamber perfusion, but none within the preceding 5–6 weeks. All had biomicroscopically normal anterior segments, free of cells and flare, at the time of the present experiments. Baseline facility in both eyes was determined for 35 min. This was followed by topical administration of 100 μg of R-PIA to the superior cornea of the treated eye and 15% DMSO to the control eye. The lower eyelid was lifted several times immediately after each drop to ensure good drug/vehicle contact with the cornea. The reservoirs were closed for 3 hr after drug administration to minimize resistance washout (Kaufman, True-Gabelt and Erickson-Lamy, 1988) and then opened for post-drug outflow facility determination for 35 min.

Slit Lamp Examination

Slit lamp biomicroscopy was performed by a trained ophthalmologist before drug administration, during single-dose IOP experiments at 3 and 6 hr after dosing, and during multiple dose experiments at 2, 6 and 9 hr after the first dose and at 2, 6, 9, and 24 hr after the seventh dose. The integrity of the corneal epithelium, and the presence or absence of flare (protein) in the anterior chamber, an indication of blood-aqueous barrier breakdown, were noted.

Data Analysis

Data are presented as mean $\pm$ S.E.M. for n eyes or animals. Pre- or post-drug treated vs. contralateral

control; post-drug or post-vehicle vs. ipsilateral baseline; and baseline corrected post-drug treated vs. control comparisons were made, using the 2-tailed paired t-test for ratios vs. 1·0 or differences vs. 0·0. Comparisons between monkey ages in different treatment groups were made, using the 2-tailed unpaired t-test for differences and the χ^2-test for age distribution (< 3 yr vs. ⩾ 3 yr).

3. Results

Intraocular Pressure

The following text refers to baseline- and control eye-adjusted IOP data unless otherwise stated; the absolute and adjusted data are given in Figs 1–5. A single dose of R-PIA or CHA administered unilaterally produced a biphasic effect on IOP: early ocular hypertension at 30 min, followed by ocular hypotension from 2–6 hr. With R-PIA, maximum hypertension of 4·86 ± 0·84 mmHg was produced with the 250 μg dose; maximum hypotension of 2·11 ± 0·38 mmHg occurred 4 hr after the 100 μg dose (Fig. 1). With CHA, maximum hypertension of 3·50 ± 1·53 mmHg and hypotension of 3·60 ± 0·73 mmHg (at 4 hr) were produced with the 500 μg dose (Fig. 2).

On day 4 of twice daily treatment with 100 μg of R-PIA (Fig. 3) or 500 μg of CHA (Fig. 4), neither the maximum hypertension (at 30 min) nor the maximum subsequent hypotension, adjusted for baseline and control eye values, were significantly different from that on day 1. However, both drugs tended to enhance the time-dependent IOP decrease in the control eyes. The duration of ipsilateral IOP reduction was longer in the treated eyes for both agonists on day 4 than on day

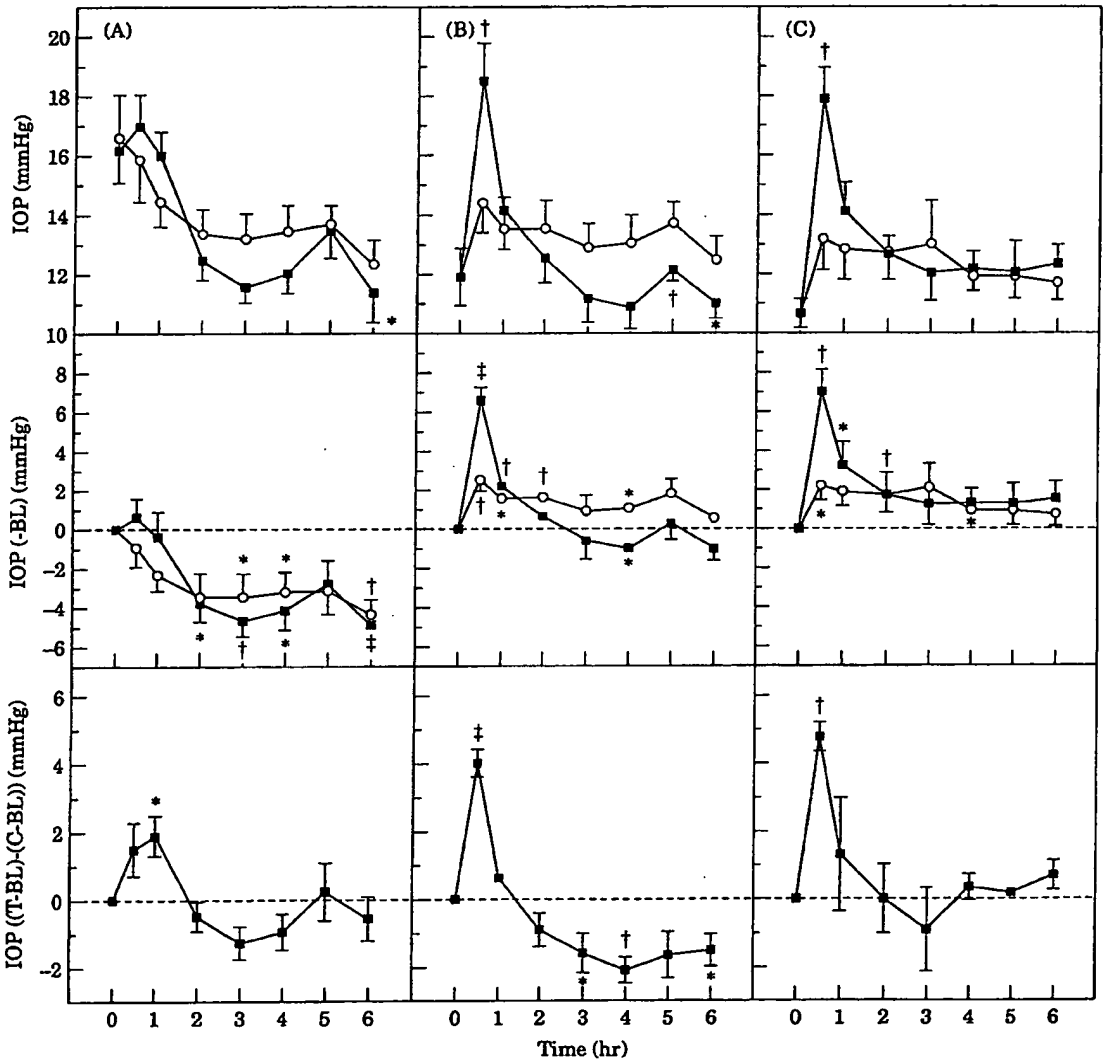

FIG. 1. Effect of a single topical dose of R-PIA on IOP in cynomolgus monkeys. (A) 20 μg R-PIA to treated eye; 15% DMSO to control eye (5 μl × 2, $n = 5$). (B) 100 μg R-PIA to treated eye; 15% DMSO to control eye (5 μl × 2, $n = 6$). (C) 250 μg R-PIA to treated eye; 50% DMSO to control eye (5 μl × 5, $n = 6$). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. ■, R-PIA; ○, vehicle.

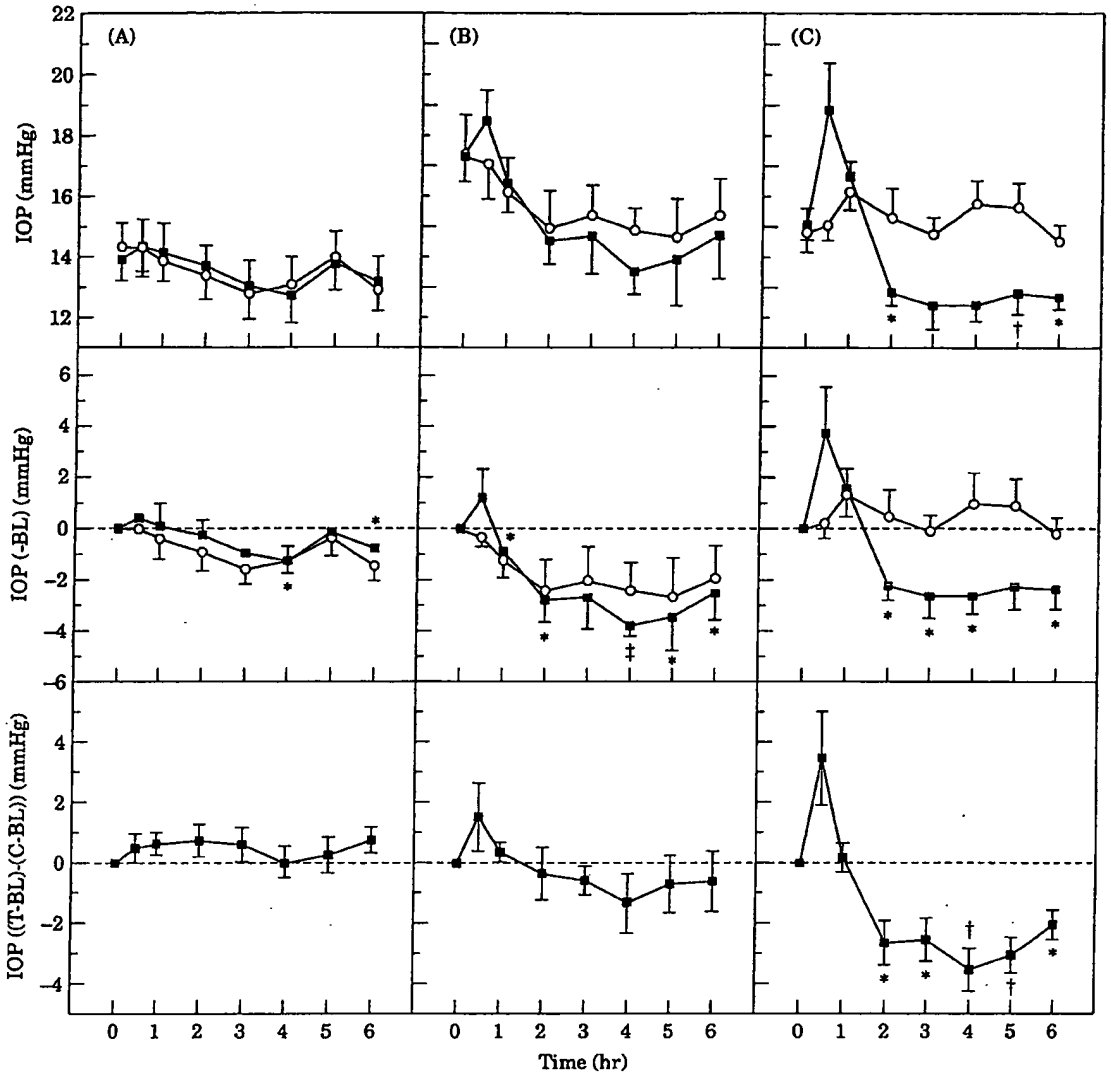

FIG. 2. Effect of a single topical dose of CHA on IOP in cynomolgus monkeys. (A) 20 μg CHA to treated eye; 15% DMSO to control eye (5 μl × 2, $n = 5$). (B) 100 μg CHA to treated eye; 15% DMSO to control eye (5 μl × 2, $n = 7$). (C) 500 μg CHA to treated eye; 25% DMSO to control eye (5 μl × 4, $n = 5$). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. ■, CHA; ○, vehicle.

1, compared to same-day ipsilateral baseline; compared to baseline- and control eye-corrected values, day 4 duration exceeded day 1 duration for R-PIA but not CHA. The maximum hypotension in R-PIA-treated eyes compared to baseline was greater in magnitude on day 4 than on day 1; IOP in the contralateral control eyes was also reduced significantly on day 4, but the effect was smaller in magnitude than in drug-treated eyes.

In bilateral agonist + unilateral antagonist experiments, pretreatment with the A_2 antagonist DMPX (320 μg) substantially attenuated the initial agonist-induced hypertension at 0·5 hr. The IOP at 0·5 hr in the agonist + antagonist-treated eye was significantly lower than in the contralateral agonist-only treated eye (1·69 ± 1·07 mmHg vs. 5·06 ± 0·70 mmHg in R-PIA–DMPX protocol; 1·97 ± 0·59 mmHg vs. 4·81 ± 0·93 mmHg in CHA–DMPX protocol; $P < 0.01$). IOP in the DMPX-pre-treated eye tended to remain lower (albeit not significantly) throughout the experiment. DMPX alone had no significant effect on IOP (Fig. 5).

Aqueous Humor Flow

AHF did not generally differ significantly between treated and control eyes following either R-PIA or CHA (Table I). In the bilateral agonist–unilateral antagonist experiment, AHF was decreased by 24–31% ($P < 0.05$) in the DMPX + CHA-treated eyes and by 9–15% ($P > 0.05$) in the contralateral CHA only-treated eyes, compared to no-drug baselines. AHF in the DMPX + CHA-treated eyes generally averaged ~ 10% less than in contralateral CHA only-treated

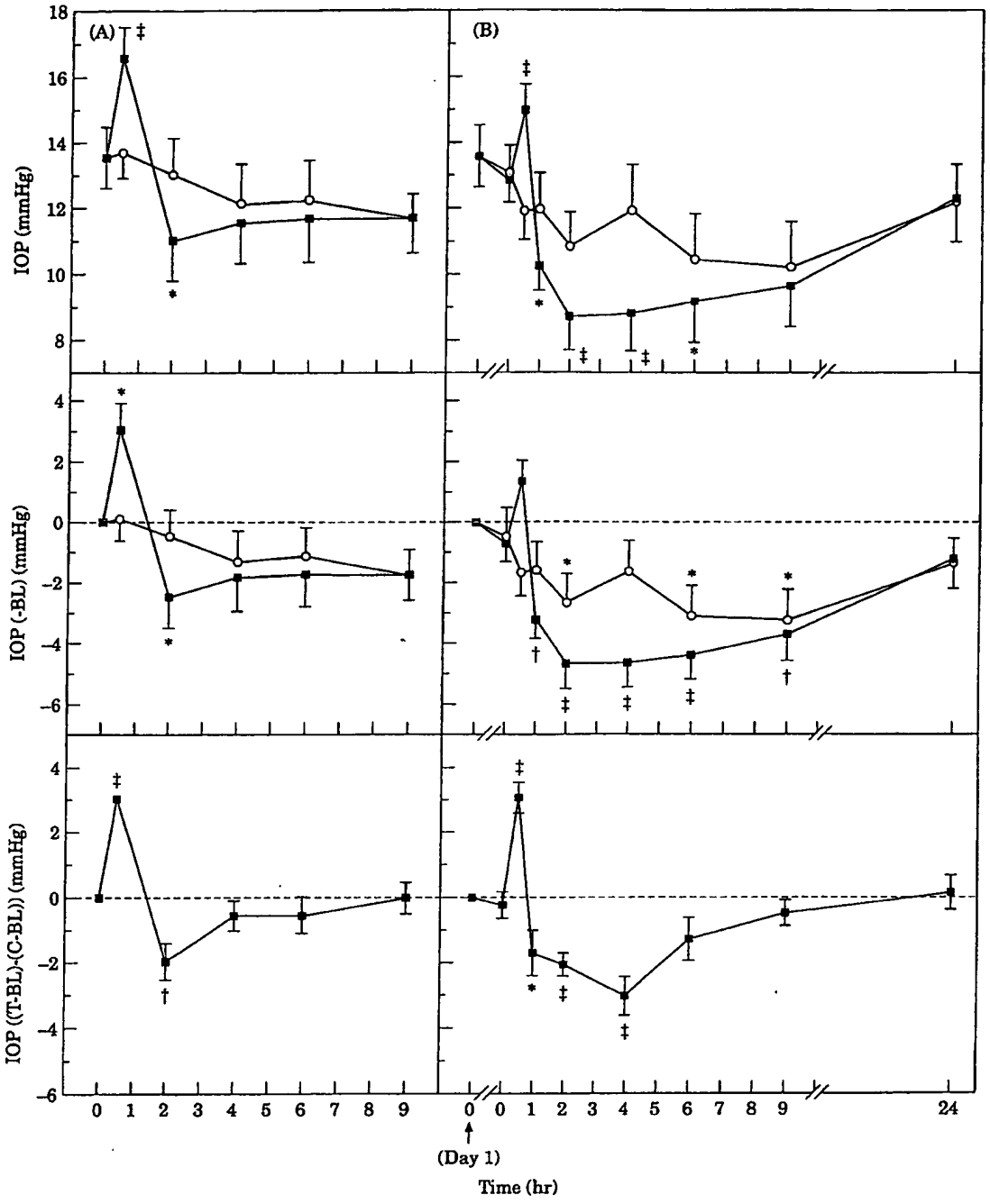

FIG. 3. Effect of twice daily topical (100 μg × 7) R-PIA doses on IOP in cynomolgus monkeys $n = 9$. (A) Day 1: one dose of 100 μg R-PIA (5 μl × 2) to treated eye; 15% DMSO (5 μl × 2) to control eye; (B) Day 4: seventh dose of 100 μg R-PIA (5 μl × 2) to treated eye; 15% DMSO (5 μl × 2) to control eye (twice daily). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. ■, R-PIA; ○, vehicle.

eyes, whether or not adjusted for the respective no-drug baselines, but the ratio did not differ significantly from 1·0 ($P > 0.05$) over any time interval (Table II).

Outflow Facility

Topical administration of 100 μg of R-PIA increased outflow facility relative to baseline by 71% compared to contralateral control eyes (corrected for perfusion-induced resistance washout, as estimated from the post-drug/pre-drug facility ratio in the control eyes) at 3–3·5 hr (Table III).

Slit Lamp Examination

Slit lamp biomicroscopy 3 hr after a single topical dose of CHA (100 μg) revealed very mild flare (1+ on a scale of 4) in the anterior chambers of the treated

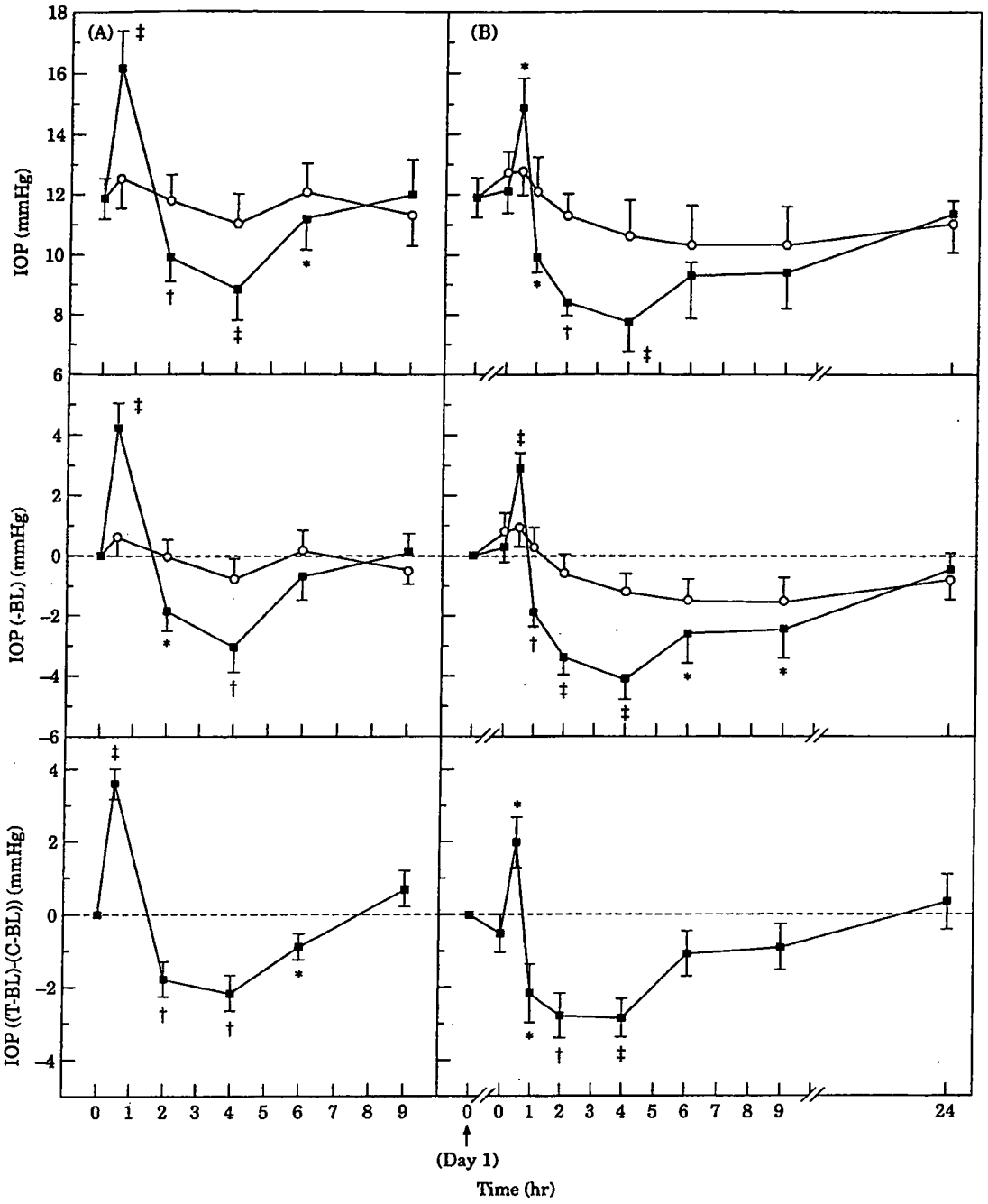

FIG. 4. Effect of twice daily topical (500 μg × 7) CHA doses on IOP in cynomolgus monkeys $n = 9$. (A) Day 1: one dose of 500 μg CHA (5 μl × 4) to treated eye; 25% DMSO (5 μl × 4) to control eye; (B) Day 4: seventh dose of 500 μg CHA (5 μl × 4) to treated eye; 25% DMSO (5 μl × 4) to control eye (twice daily). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. ■, CHA; ○, vehicle.

and control eyes of 1 out of 7 monkeys, which disappeared (treated eye) or decreased significantly (control eye) by 6 hr. Slit lamp examination also revealed 1+ flare following 250 μg R-PIA in 3 of 5 treated eyes, which disappeared completely by 6 hr. Mild superficial punctate corneal epitheliopathy was observed bilaterally during all single or multiple topical treatment experiments, as is typical with repeated tonometry. There were no apparent differences between treated and control eyes, and between single and multiple treatments. No abnormal findings, except the bilateral punctate keratopathy were observed during the DMPX/agonists or DMPX alone IOP experiments. The presence or absence of external ocular vasodilation was difficult to discern in the heavily pigmented monkey conjunctiva.

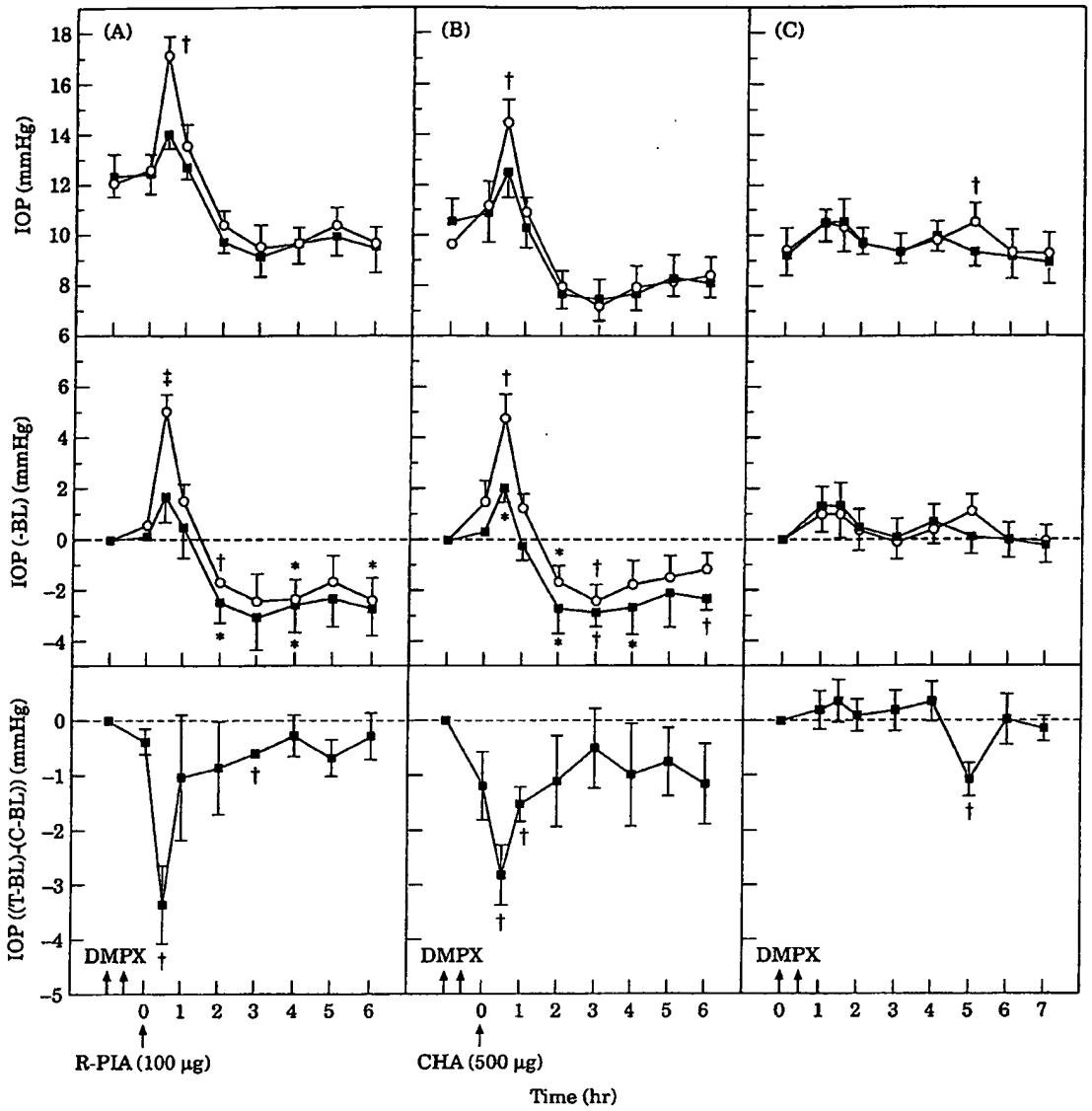

FIG. 5. Effect of two topical (160 μg × 2) DMPX doses on IOP in cynomolgus monkeys $n = 6$. (A) 160 μg DMPX (8 μl × 4) to treated eye 30 and 60 min before 100 μg R-PIA (5 μl × 2); 20% DMSO (8 μl × 4) to control eye 30 and 60 min before 100 μg R-PIA (5 μl × 2); ■, DMPX + R-PIA, ○, R-PIA. (B) 160 μg DMPX (8 μl × 4) to treated eye 30 and 60 min before 500 μg CHA (5 μl × 4); 20% DMSO (8 μl × 4) to control eye 30 and 60 min before 500 μg CHA (5 μl × 4); ■, DMPX + CHA; ○, CHA. (C) 160 μg DMPX (8 μl × 4) to one eye at hr 0 and hr 0·5; 20% DMSO (8 μl × 4) to opposite eye at hr 0 and hr 0·5; ■, DMPX; ○, vehicle. IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: $^*P < 0.05$; $^\dagger P < 0.01$; $^\ddagger P < 0.001$.

4. Discussion

The IOP response to the adenosine agonists R-PIA and CHA in cynomolgus monkeys, reported for the first time in this study, was generally similar to that previously reported in rabbits (Camras et al., 1994; Crosson, 1992, 1995). In rabbits treated topically with R-PIA (Crosson, 1992), ocular hypertension of 3·5 mmHg at 0·5 hr was found after a 500 μg dose and was followed by a significant IOP reduction (5–8 mmHg), compared to vehicle-treated rabbits. At lower R-PIA doses, Crosson's rabbits exhibited no ocular hypertension but the reduction of IOP was still significant. Camras et al. (1994) administered 200 μg of R-PIA topically to rabbits and produced an 8·4 mmHg IOP rise at 0·5 hr and a maximum IOP reduction of 5·8 mmHg at 5 hr, compared with contralateral control eyes. In rabbits treated topically with CHA (Crosson, 1995), an initial hypertensive response of 2·3 ± 0·8 mmHg at 0·5 hr was induced after the administration of the 500 μg dose, followed by a significant reduction in IOP of 4–6 mmHg from 2–6 hr. In our monkeys, both R-PIA and CHA had a biphasic effect on IOP, producing ocular hypertension of ~ 4 mmHg within 30 min, followed by an IOP reduction of 2–3·5 mmHg from 2–6 hr with a single dose of 100 μg R-PIA or 500 μg CHA.

Although R-PIA and CHA are relatively selective

TABLE I

Aqueous flow after topical 100 μg R-PIA or 500 μg CHA in monkeys

	Hours 0·5–1·5			Hours 0·5–3·0		
	Trt	Cont	T/C	Trt	Cont	T/C
R-PIA	1·25±0·12	1·15±0·13	1·15±0·12	1·32±0·14	1·16±0·13	1·16±0·06*
CHA	0·76±0·14	0·88±0·10	0·88±0·14	1·08±0·13	1·02±0·16	11·12±0·12
	Hours 3·5–6·0			Hours 0·5–6·0		
	Trt	Cont	T/C	Trt	Cont	T/C
R-PIA	1·37±0·29	1·25±0·19	1·07±0·10	1·41±0·27	1·32±0·21	1·05±0·08
CHA	1·36±0·25	1·42±0·22	0·94±0·04	1·28±0·19	1·25±0·17	1·02±0·04

Topical administration of 100 μg R-PIA or 500 μg CHA in treated eyes and vehicle in control eyes. Flow data are mean±S.E.M. μl min^{-1}, with R-PIA, $n = 8$ during hr 0·5–3·0, $n = 5$ during hr 3·5–6·0; With CHA, $n = 5$; $^*P < 0·05$ by the 2-tailed paired t-test for ratios ≠ 1·0. Times are hr post-treatment.

TABLE II

Aqueous flow after topical 500 μg CHA or 320 μg DMPX + CHA in monkeys

	Hours 0·5–1·5			Hours 0·5–3·0		
	Trt	Cont	T/C	Trt	Cont	T/C
BL	1·32±0·15	1·17±0·15	1·17±0·12	1·41±0·15	1·37±0·18	1·05±0·05
Exp	0·85±0·08	1·01±0·15	0·99±0·24	1·03±0·09	1·13±0·12	0·92±0·05
Exp/BL	0·69±0·11*	0·88±0·11	0·90±0·26	0·75±0·06†	0·85±0·06	0·89±0·05
	Hours 3·5–6·0			Hours 0·5–6·0		
	Trt	Cont	T/C	Trt	Cont	T/C
BL	1·58±0·15	1·63±0·25	1·07±0·17	1·60±0·16	1·56±0·21	1·06±0·07
Exp	1·20±0·14†	1·38±0·14	0·87±0·06	1·16±0·10	1·28±0·12	0·92±0·04
Exp/BL	0·76±0·03†	0·91±0·10	0·88±0·10	0·73±0·03†	0·86±0·08	0·88±0·05

Topical administration of 320 μg DMPX and 500 μg CHA in treated eyes and 500 μg CHA in control eyes. BL, baseline flow (average of two separate baselines one week before and seven weeks after drugs); Exp, flow after drugs. Flow data are mean±S.E.M. μl min^{-1}, $n = 6$; $^*P < 0·05$; †$P < 0·01$ by the 2-tailed paired t-test for ratios ≠ 1·0. Times are hr post-treatment.

TABLE III

Effect of a single topical 100 μg R-PIA dose on outflow facility

	Outflow facility			
	Treated	Control	T-C	T/C
BL	0·336±0·070	0·369±0·080	−0·033±0·058	1·023±0·122
R-PIA	0·628±0·163	0·398±0·091	0·230±0·083*	1·644±0·162†
R-PIA/BL	1·839±0·143	1·129±0·100	0·710±0·157†	1·712±0·183†

Facility data are mean±S.E.M. (μl min^{-1} $mmHg^{-1}$); $n = 9$; BL = baseline. Ratio ≠ 1·0, difference ≠ 0·0 by the 2-tailed paired t-test: $^*P < 0·05$, †$P < 0·01$.

adenosine A_1 agonists (Bruns, Lu and Pugsley, 1986), studies in rabbits have demonstrated that at the topical doses used in this study these agents can activate both adenosine A_1 and A_2 receptors in the eye (Crosson, 1992, 1995; Crosson and Gray, 1996). In both Crosson and Gray's, (1996) rabbit study and our monkey study, pretreatment with the adenosine A_2 antagonist DMPX substantially blocked the early

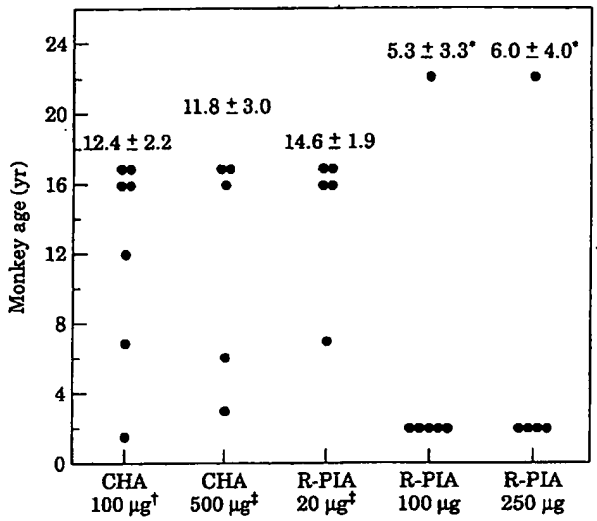

FIG. 6. Monkey ages in different treatment groups. Each point represents one animal. Group data are mean ± S.E.M. yr. Age data for the 20 μg CHA group and for one monkey in the 250 μg R-PIA group not available. * $P < 0.001$ by the 2-tailed unpaired t-test for age differences between 100 μg R-PIA or 250 μg R-PIA group and 20 μg R-PIA, 100 μg CHA or 500 μg CHA group. † $P < 0.0$, ‡ $P < 0.025$ by the χ^2-test for age distribution (< 3 yr vs. ≥ 3 yr) between 100 μg CHA, 500 μg CHA or 20 μg R-PIA group and 100 μg R-PIA or 250 μg R-PIA group.

hypertension without attenuating the subsequent hypotension, indicating that the hypertensive phase is associated with A_2 receptor stimulation. In our monkeys, IOP in the DMPX-pre-treated eye tended to remain lower throughout the experiment, suggesting that A_2 receptor stimulation may not only cause the initial hypertension, but also induce a functional antagonism of the A_1 receptor-mediated hypotension similar to that described in rabbits (Crosson, 1995). Crosson (1992) also demonstrated that the adenosine A_1 antagonist CPT can inhibit the hypotensive response to R-PIA. An A_1 antagonist was not employed in the current study, but absent attenuation of the hypotension by the A_2 antagonist, it seems reasonable to attribute the hypotension to A_1 receptor stimulation.

R-PIA and CHA are close structural analogs exhibiting similar pharmacokinetic profiles and approximately equal activity at the receptors (Crosson, 1992, 1995). In Crosson's rabbits, the hypertensive and hypotensive responses to both R-PIA and CHA were dose-dependent. In our monkeys, the hyper- and hypotensive responses to CHA both tended to be dose-dependent. However, in animals treated with R-PIA dose-dependence was observed only for the early rise in IOP. Although significant IOP reduction occurred with the mid-range dose of R-PIA (100 μg), a hypotensive response was not evident for the highest R-PIA dose (250 μg) evaluated, perhaps related to the age of the monkeys. Monkeys receiving 100 μg and 250 μg of R-PIA were primarily 2-year olds and much younger than those in the other groups (Fig. 6). Such young monkeys usually have lower baseline IOP (Fig. 1), so that outflow facility enhancement or AHF reduction will have less IOP effect than at higher starting IOP (Kaufman, 1985). Additionally, the levels of adenosine A_2 receptor mRNA and adenosine A_2 binding sites are reduced by 32% and 20% respectively in old compared to young rat striatum (Schiffmann and Vanderhaeghen, 1993), suggesting that the density of adenosine A_2 receptors is higher in younger animals. This conclusion appears to be consistent with the data presented in Figs 1 and 2 where the hypertensive response to the younger monkeys treated with R-PIA is much larger than the corresponding response to CHA in older animals. In rabbits and cats of equal age no difference in hypertensive response is observed when CHA and R-PIA are compared (Crosson, unpublished results). Since the net change in IOP likely results from a combination of the hypertensive and hypotensive responses, a larger hypertensive response to 100 or 250 μg R-PIA in the two younger animal groups may have blunted the subsequent IOP reduction and obscured its dose-dependence (Fig. 1).

In our study, no significant differences were observed between the hypertensive responses to either agonist on day 4 and day 1. The ipsilateral hypotensive response following 100 μg R-PIA increased in magnitude on day 4 compared to day 1. However, the control eyes also exhibited a hypotensive response on day 4, reducing the apparent magnitude and duration of the baseline- and control eye-adjusted change. The IOP reduction in the contralateral control eyes was greater on day 4 than day 1, suggesting it was not due to ketamine anesthesia alone (Hahnenberger, 1976; Gabelt et al., 1994) but perhaps to a contralateral response to the A_1 effect of the adenosine agonists (Crosson, 1995).

Investigation of the physiological mechanism of the adenosine agonist-induced ocular hypertension or hypotension in rabbits has yielded conflicting results. Camras et al. (1994) found a transient increase in AHF by fluorophotometry during the hypertensive phase following a single 200 μg dose of R-PIA, but no effect on AHF during the hypotensive phase compared to contralateral control eyes. Crosson and Gray (1996) demonstrated that topical administration of 165 μg of the adenosine A_2 agonist CV-1808 was associated with increased aqueous humor flow and protein concentration. Braunagel, Xiao and Chiou (1988) confirmed that adenosine produced retinal and choroidal vasodilatation in rabbits. Anterior uveal vasodilatation could induce breakdown of the blood-aqueous barrier and consequently increased IOP (Funk, Wagner and Rohen, 1992; Krootila et al. 1992). However, at lower doses of CV-1808 IOP rose without an increase in aqueous humor protein, suggesting that two independent mechanisms are responsible for the adenosine A_2-induced rise in IOP (Crosson and Gray, 1996). In addition, Crosson (1995) demonstrated that R-PIA (165 μg) and CHA (165 μg)

reduced AHF by 37% and 35% respectively during the early part of hypotensive response when compared to other vehicle-treated rabbits.

In our monkeys, there was little evidence for an early increase in AHF after 100 μg R-PIA or 500 μg CHA, and certainly not one sufficient to account for the early ocular hypertension. At high doses of adenosine A_2 agonists the blood-aqueous barrier may break down secondary to hemodynamic changes in the ciliary body, perhaps producing a minimal increase in AHF while at low doses of A_2 agonists or with relatively selective A_1 agonists such as R-PIA or CHA, the transient IOP rise may be associated with vascular expansion in the ciliary body without increased AHF. There was also little evidence for a subsequent decrease in AHF during the hypotensive phase. However, when compared to baselines obtained on separate occasions, AHF was significantly decreased by 24–31% in CHA + DMPX-treated eyes (Table II). It may be that A_1 receptor stimulation slightly decreased ipsilateral AHF but the apparent effect was minimized by (1) a contralateral A_1 receptor-related inhibition of AHF and (2) an ipsilateral A_2 receptor-related effect. Overall these data suggest that in monkeys adenosine agonists at most have only a modest effect on AHF.

In rabbits, total outflow facility (2-level constant pressure perfusion) after 200 μg R-PIA was significantly increased during the hypotensive phase (Camras et al., 1994). Perfusion of the anterior chamber of monkey eyes with drug free Bárány's mock aqueous produces a time-dependent facility increase, presumably due to washout or resistance-producing extracellular material from the trabecular meshwork (Kaufman et al., 1988). In the present experiments, the resistance washout was minimized by closing the reservoirs after baseline facility measurements until beginning post-drug facility measurements. The 71% increase in outflow facility 3–3·5 hr following topical R-PIA (relative to the control eye and adjusted for resistance washout) in our monkeys (Table III) is sufficient to produce the observed IOP reduction, according to the modified Goldmann equation (Kaufman, 1985). Our control eye post-drug/pre-drug facility ratio of 1·13 is consistent with the ~ 15% 'washout effect' seen in other comparably-timed perfusions without drugs (Kaufman et al., 1988), indicating the absence of a contralateral drug effect on facility, contralateral effects on IOP not withstanding.

We saw little apparent ocular toxicity following topical R-PIA, CHA, DMPX, or DMSO vehicle by slit lamp biomicroscopy. The mild superficial punctate keratopathy was consistent with repeated tonometry. The presence or absence of external ocular vasodilation was not discerned because of the heavily pigmented monkey conjunctiva. Mild transient anterior chamber flare was seen only in some 2 yr-old monkeys, mainly in eyes treated with the higher dose of R-PIA. Such flare indicates breakdown of the blood-aqueous barrier, as in Crosson and Grays (1996) rabbits. Because the younger monkeys may have more A_2 receptors (Schiffmann and Vanderhaeghen, 1993) whose activation may produce more aqueous humor protein (Crosson and Gray 1996) and resultant ocular hypertension, the biomicroscopic findings support the hypothesis that activation of A_2 receptors may have blunted the subsequent ocular hypotension especially in the younger animals.

In summary, these results demonstrate that relatively selective adenosine A_1 agonists produce a biphasic change in IOP following topical administration to monkeys: early hypertension followed by prolonged hypotension. The hypertensive and hypotensive phases are associated with activation of adenosine A_2 and A_1 receptors respectively. The IOP decrease appears to result primarily from an increase in outflow facility.

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